

New Zealand sheep with scrapie-susceptible PrP genotypes succumb to experimental challenge with a sheep-passaged scrapie isolate (SSBP/1)

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Scrapie does not occur in New Zealand (NZ), although PrP gene alleles associated with susceptibility to the disease are found at relatively high frequencies in NZ sheep. The hypothesis that scrapie is a genetic disease of sheep is thus unlikely to be true. To confirm that NZ sheep are actually susceptible to scrapie infection, NZ sheep of various PrP genotypes were challenged by subcutaneous inoculation with a sheep-passaged scrapie isolate (SSBP/1). Showing similar PrP genetics to that seen in UK sheep, all NZ sheep carrying the VRQ PrP allele developed clinical signs typical of scrapie, with characteristic neurodegenerative changes and PrP^{Sc} evident on histopathological examination of their brains and lymphoid tissues. The incubation periods recorded in NZ sheep were generally shorter than those found in UK sheep. The results confirm that New Zealand sheep are as susceptible as their UK counterparts to experimental scrapie infection by subcutaneous inoculation.

Scrapie is a member of the group of diseases termed transmissible spongiform encephalopathies (TSEs), which are characterized by progressive neurodegenerative changes in the brain, leading to neurological impairment and eventually death. It has been recognized for many years that resistance and susceptibility to scrapie in sheep are genetically controlled (Dickinson *et al.*, 1968) via polymorphisms of the sheep PrP (prion protein) gene (Hunter, 1997). In many breeds, e.g. Cheviot and Swaledale, susceptibility to scrapie is linked to valine at codon 136, with VRQ/VRQ homozygotes (the letters stand for the amino acids encoded at codons 136, 154 and 171 respectively) being the most susceptible genotype (Goldmann *et al.*, 1991; Hunter *et al.*, 1993, 1996). In other

breeds, e.g. Suffolk, the VRQ PrP allele is extremely rare and instead animals with the ARQ/ARQ genotype are at most risk of scrapie, although not all animals with this genotype develop the disease (Hunter *et al.*, 1997a). Certain PrP gene alleles have a protective effect, e.g. ARR, so that animals with the VRQ/ARR genotype very rarely develop natural scrapie (Belt *et al.*, 1995; Hunter *et al.*, 1996) despite being fully susceptible to experimental challenge. ARR/ARR homozygotes appear to be resistant to scrapie, with only one reported case worldwide in a sheep of this genotype (Ikeda *et al.*, 1995). The strong association between certain genotypes and susceptibility to disease, in particular the high incidence of scrapie in VRQ/VRQ animals in the UK and Europe, have led some to propose that scrapie is a genetic rather than an infectious disease (Ridley & Baker, 1995). New Zealand (NZ) and Australia are countries that are entirely free of scrapie, yet analysis of the PrP genotypes from sheep breeds in these countries demonstrated that scrapie-susceptible genotypes did occur at relatively high frequencies (Hunter *et al.*, 1997b; Hunter & Cairns, 1998; Bossers *et al.*, 1999). In addition, two of twenty Suffolk sheep imported from NZ into a scrapie-infected flock in the USA succumbed to the disease, as did 39% of their progeny (Hourrigan *et al.*, 1979). Together these findings suggest that the most likely explanation for the absence of scrapie in Australia and NZ is that the infectious agent is absent. However, since information is lacking on the PrP genotypes and incubation periods in the NZ Suffolks imported into the USA, it is still possible that unrecognized genetic factors that have some protective effect against scrapie may have been selected in sheep breeds in NZ and Australia. The purpose of this study was therefore to determine whether NZ sheep with scrapie-susceptible PrP genotypes succumb to experimental challenge with scrapie.

The sheep were Cheviots imported from NZ by the Department for Environment, Food and Rural Affairs (DEFRA; formerly the Ministry of Agriculture, Fisheries and Food), or were the offspring of a scrapie-free breeding flock (the DEFRA/SF flock) established from the imported animals. They were held in strict isolation before being transported to IAH,

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Table 1. Distribution of PrP^{Sc} in the lymphoid tissues of Cheviot sheep inoculated with SSBP/1 by the subcutaneous route

Tissues: PSLN, prescapular lymph node; MLN, mesenteric lymph node; IPP, ileal Peyer's patch; JPP, jejunal Peyer's patch. Positives: the number of sections in which positive staining for PrP^{Sc} could be detected over the number of readable sections. Scores give an indication of the percentage of positive follicles per section (1 = 1–24%; 2 = 25–49%; 3 = 50–74%; 4 = 75–100%) and a subjective estimate of the intensity of the staining (from +/- = marginal to +++ = strong). In some groups, there was considerable variability between the scores from different animals. The intensity of the staining was not scored in the Peyer's patches, as most had small focal intensely stained deposits of PrP^{Sc}, rather than the diffuse reticular pattern of staining seen in other lymphoid tissues.

Tissue	VRQ/VRQ		VRQ/ARQ		VRQ/ARR	
	Positives	Score	Positives	Score	Positives	Score
PSLN	5/5	1 (++)	5/5	1 (+)	3/5	1 (+)
MLN	4/4	1 (+)	2/5	1 (+)	1/5	1 (+)
Tonsil	5/5	2 (++)	5/5	1 (+)	4/5	1 (+/-)
Spleen	5/5	2 (++)	4/5	1 (+)	1/5	1 (+)
IPP	5/5	2	0/2	–	0/4	–
JPP	5/5	3	3/5	1	1/5	3

Compton. Six groups of five DEFRA/SF animals 6–11 months of age and with PrP genotypes VRQ/VRQ, VRQ/ARQ, VRQ/ARR, ARQ/ARQ, ARQ/ARR and ARR/ARR, were inoculated subcutaneously with SSBP/1 (2 ml 10% brain homogenate). This dose is known to induce scrapie in 100% of inoculated UK Cheviots carrying the VRQ allele with incubation periods that are not related to age (J. D. Foster & W. Goldmann, unpublished). In a related experiment Poll Dorset sheep (VRQ/ARQ and VRQ/ARR) from a flock (PD1) imported from NZ and maintained free of scrapie (Hunter & Cairns, 1998) were also inoculated subcutaneously with SSBP/1.

All animals were housed under strict isolation and fed on a diet of dried lucerne pellets and hay, with free access to a mineral supplement. Care was taken to source the lucerne and hay from farms/land that had been free of livestock for at least 20 years and to ensure that the diet did not contain any animal protein.

When clinical signs consistent with scrapie were clearly present, the animals were humanely destroyed according to Home Office regulations. The brains were removed at necropsy and divided longitudinally. One portion was fixed in neutral buffered formalin (NBF) and processed for histopathological examination according to standard protocols. Samples of lymphoid tissues (spleen, tonsil, prescapular lymph node, mesenteric lymph node, Peyer's patch) were also taken and fixed in NBF. Immunohistochemical staining with the avidin–biotin complex system was carried out on paraffin-embedded sections using a method involving hydrated autoclaving, based on the technique described for detection of PrP^{Sc} by Haritani *et al.* (1994). Immunostaining for PrP^{Sc} in the brain

was carried out using a polyclonal antibody raised in rabbits to amino acids 217–231 of the bovine PrP sequence (R482) and also with R521 (van Keulen *et al.*, 1995), which provide good staining of sheep scrapie-affected brains from a range of breeds and genotypes. Immunostaining for PrP^{Sc} in the lymphoid tissues was carried out using the mouse monoclonal antibody FH11 (Foster *et al.*, 1996).

All DEFRA/SF Cheviot sheep in the PrP genotype groups VRQ/VRQ, VRQ/ARQ and VRQ/ARR developed clinical signs suggestive of scrapie at various intervals following inoculation with SSBP/1 while ARQ/ARQ, ARQ/ARR and ARR/ARR Cheviots remain alive and healthy at the time of writing (1108, 1110 and 729 days post-inoculation, respectively). It is intended that animals in this experiment are allowed to survive for lifespan because although expected to be resistant to SSBP/1 challenge, this needs to be confirmed. In the scrapie-affected VRQ sheep, the first sign to be observed was pruritus, manifesting as rubbing of the hindquarters and flanks (often accompanied by nibbling movements and licking the lips) and/or nibbling and biting of the lower limbs. All the animals also showed ataxia and incoordination, initially affecting the hind limbs, but progressing to involve the forelimbs in some cases. This appeared to be associated with proprioceptive deficits, exhibited by wide base stance, stumbling, swaying, greater abduction/adduction of the hind limbs and a tendency to fall when turning sharply. There were no marked abnormalities of mentation or behaviour, e.g. increased nervousness, aggression or hyperaesthesia, although some animals appeared mildly depressed. These clinical signs are consistent with those previously reported in sheep infected with SSBP/1, where pruritus, incoordination of gait and

weight loss were the predominant clinical signs (Wilson *et al.*, 1950; Dickinson *et al.*, 1968).

The diagnosis of scrapie was confirmed by the demonstration of mild, though characteristic, lesions on histological examination of the brain and by immunostaining for PrP^{Sc}. The patterns of vacuolation and PrP^{Sc} accumulation were similar in all three genotype groups. Sparse or mild neuropil vacuolation was present in the grey matter of a restricted range of neuroanatomical nuclei; the olives, ventral thalamic nucleus, the dorso-medial thalamic nucleus, caudate nucleus and accumbens. This limited range of vacuolation is similar to that previously found with this experimental scrapie strain in UK Cheviot and Swaledale sheep (Foster *et al.*, 1996).

Immunostaining indicated widespread disease-specific PrP^{Sc} accumulation in brain. In addition to the vacuolated sites mentioned above, PrP^{Sc} accumulations were also found in dorsal brainstem nuclei (including the dorsal motor nucleus of the vagal nerve), cerebellar, midbrain and cerebrocortical sites, which did not show vacuolation. Several patterns of disease-specific PrP^{Sc} accumulation were found including intraneuronal PrP^{Sc} accumulation, stellate accumulations (thought to be associated with astrocytes) and diffuse granular patterns of immunolabelling. These patterns of PrP^{Sc} accumulation in brain are also typical of natural sheep scrapie (van Keulen *et al.*, 1995).

Immunostaining was also performed on sections of pre-scaphular lymph node (PSLN), mesenteric lymph node (MLN), tonsil, spleen, jejunal and ileal Peyer's patch (JPP and IPP respectively) from each animal. In general, the pattern of PrP^{Sc} deposition was similar to that seen in natural scrapie, i.e. diffuse reticular staining within follicular germinal centres characteristic of accumulation on follicular dendritic cells (FDCs). There were clear differences between the genotypes in the distribution and relative levels of PrP^{Sc} accumulation (see Table 1). In VRQ/VRQ sheep, PrP^{Sc} was consistently detected in all the lymphoid tissues of every animal examined, and the percentage of positive follicles and intensity of staining were higher than in the other genotypes. In VRQ/ARQ sheep, PrP^{Sc} was most consistently detected in the PSLN (5/5 animals), tonsil (5/5 animals) and spleen (4/5 animals). The distribution of PrP^{Sc} was much more restricted, and the staining much weaker, in VRQ/ARR sheep than in the other genotypes, although 4/5 animals showed weak staining in the tonsil and 3/5 were positive in the PSLN. In general, these observations are similar to natural scrapie cases. However, the intensity of the staining was weaker, and the distribution of PrP^{Sc} in VRQ/ARQ sheep was more restricted, than would be expected in naturally infected animals. In natural cases, PrP^{Sc} is usually widely distributed in lymphoid tissues of both VRQ/VRQ and VRQ/ARQ animals by the time clinical signs develop. Interestingly, in the rare natural cases of scrapie in VRQ/ARR sheep, PrP^{Sc} is not found in lymphoid tissues (van Keulen *et al.*, 1996; Andréoletti *et al.*, 2000; M. Jeffrey, unpublished), but all of the SSBP/1-infected sheep showed

Table 2. Incubation periods of experimental scrapie in UK Cheviots and NZ-derived Cheviots and Poll Dorsets inoculated with SSBP/1 by the subcutaneous route

Flock and breed/genotype	Incubation period in days (mean \pm SD)	Survival period (days)
DEFRA/SF* – Cheviot		
VRQ/VRQ	150 \pm 11 (n = 5)	–
VRQ/ARQ	256 \pm 39 (n = 5)	–
VRQ/ARR	259 \pm 9 (n = 5)	–
ARQ/ARQ	–	> 1000 (n = 5)
ARQ/ARR	–	> 1000 (n = 4)
ARR/ARR	–	> 700 (n = 5)
NPU† – Cheviot		
VRQ/VRQ	180 \pm 20 (n = 7)	–
VRQ/ARQ	269 \pm 76 (n = 17)	–
VRQ/ARR	352 \pm 63 (n = 23)	–
ARQ/ARQ	–	998–2330 (n = 3)
ARQ/ARR	–	820–4250 (n = 17)
ARR/ARR	–	1150–2720 (n = 4)
PD1* – Poll Dorset		
VRQ/ARQ	218 \pm 7 (n = 3)	–
VRQ/ARR	243 \pm 2 (n = 3)	–

* Sheep of NZ origin.

† UK sheep, including data previously published by Goldmann *et al.* (1994).

positive staining, albeit weak, in at least one of the tissues examined. A similar study of lymphoid tissues from SSBP/1-inoculated Cheviots from the IAH Neuropathogenesis Unit (NPU) flock is still in progress, and a full comparison of the pathogenesis of SSBP/1 scrapie in sheep inoculated by several different routes of injection will be presented in a separate publication.

The incubation periods recorded for DEFRA/SF NZ Cheviot and PD1 Poll Dorset sheep in the three PrP genotype groups that showed clinical signs are given in Table 2. Incubation periods for SSBP/1-inoculated NPU Cheviots are also presented for comparison. The NPU Cheviot results are pooled from several separate experiments and include those published in a previous study by Goldmann *et al.* (1994). Incubation periods for the NZ-derived scrapie-free sheep were comparable, or even shorter, than those recorded for the NPU Cheviots.

The demonstration that sheep of susceptible PrP genotypes are at least as frequent in scrapie-free countries as they are in UK flocks (Hunter *et al.*, 1997b; Hunter & Cairns, 1998; Bossers *et al.*, 1999) lent support to the hypothesis that scrapie is caused by an infection in genetically susceptible animals and that infection is not present in Australia and NZ. However not everyone was convinced by the genotype frequency results,

arguing that scrapie could still be the simple result of gene mutation – a genetic disease – if additional unknown genetic factors were acting to prevent disease occurring in scrapie-free countries (Ridley and Baker, 1998). Our results clearly demonstrate that NZ sheep are not inherently more resistant to experimental scrapie than UK animals. Although formal proof that these animals are susceptible to natural scrapie infection is still required, our findings mean that the evidence for scrapie having an infectious aetiology is now overwhelming.

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