Ovine prion protein variant $A^{136}R^{154}L^{168}Q^{171}$ increases resistance to experimental challenge with bovine spongiform encephalopathy agent

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Susceptibility and incubation periods of transmissible spongiform encephalopathies, such as scrapie in sheep, are modulated by the PrP gene. The standard model of association between ovine PrP genetics and classical scrapie susceptibility is based on PrP genotypes with respect to codons 136, 154 and 171, e.g. alanine–arginine–glutamine (ARQ). It is demonstrated here that a proline to leucine substitution in codon 168 of the ovine PrP protein gene is associated with increased resistance to experimental bovine spongiform encephalopathy (BSE) inoculation. The $ARL^{168}Q$ PrP allele was found in heterozygous $ARP^{168}Q/ARL^{168}Q$ sheep that have so far survived intravenous BSE challenge three times longer than BSE-challenged homozygous $ARP^{168}Q/ARP^{168}Q$ sheep, which develop disease in around 700 days. In contrast, the $L141F$ polymorphism does not appear to be associated with susceptibility to intravenous BSE challenge.

Transmissible spongiform encephalopathies (TSEs) are fatal, neurodegenerative diseases occurring naturally in small ruminants as scrapie and in humans mainly as Creutzfeldt–Jakob disease. A main characteristic of these diseases is the conversion of the cellular PrP$^C$ (prion) protein to the abnormal, disease-associated isoform PrP$^Sc$. Many mammals can be infected experimentally with TSEs and, in that way, it has been established that PrP protein variants modulate disease susceptibility and incubation periods (IPs) (reviewed by Bruce, 2003; Baylis & Goldmann, 2004).

The ovine PrP coding region (comprising 256 codons) is highly polymorphic, with 27 polymorphic codons described to date. The current number of published haplotypes is 43. PrP polymorphisms in codons 136, 154 and 171 are associated with length of IP after experimental exposure to various TSE agents. For European sheep breeds, the following amino acid changes in these positions are known (in single-letter amino acid code, showing on the left side the wild-type amino acid and on the right side the alternative amino acids): $A136V/T, R154H/L$ and $Q171R/H/K$. Only 11 of 36 possible haplotype combinations, e.g. $A^{136}R^{154}Q^{171}$ (ARQ), have been described to occur in sheep; the most common haplotype is ARQ (Baylis & Goldmann, 2004; Alvarez et al., 2006). Only five alleles (VRQ, ARR, AHQ, ARH and ARQ) have been tested in sheep for disease association in experimental challenges with classical scrapie or bovine spongiform encephalopathy (BSE) (Goldmann et al., 1994; O’Rourke et al., 1997; Foster et al., 2001; Houston et al., 2002, 2003; Hamir et al., 2005; Sharpe et al., 2006). It was found that the ARR allele is always associated with the longer IPs, whereas the VRQ, ARQ and AHQ alleles are associated with shorter IPs, but these differences depend on which TSE agent has been used (Goldmann et al., 1994). Susceptibility is also associated with these four PrP variants. Sheep that are highly susceptible to classical scrapie often carry the VRQ allele and resistant sheep have the ARR/ARR genotype. This resistance of ARR/ARR animals, however, can be overcome by intracerebral (i.c.) BSE challenge (Houston et al., 2003). The PrP gene association with TSEs established in experimental studies has been shown to be applicable to natural classical scrapie outbreaks in many breeds (Baylis et al., 2004) and is now part of UK and EU-wide scrapie-eradication programmes (DEFRA, 2001).

Recently, it was shown that reliance on only three PrP codon positions may not be sufficient to fully predict the susceptibility of sheep to TSE infection. Atypical scrapie has been described in several sheep flocks in several European countries. Most prominent are cases of Nor98 scrapie (Benestad et al., 2003), which were described to be associated with the $L141F$ polymorphism (Moum et al., 2005). Even genotypes most resistant to classical scrapie, such as ARR/ARR, have been shown to contain deposits of abnormal PrP protein (Buschmann et al., 2004).

Several new ovine PrP variants are discovered worldwide every year but, as their allele frequencies are usually low and as they may appear only in scrapie-free flocks, it is often
impossible to confirm their association with TSE disease. There are as yet no experimental data on association with scrapie for most of these additional haplotypes. Evidence is presented here that a P168L polymorphism on the ARQ haplotype (ARL168Q) is associated with susceptibility after experimental inoculation with the BSE agent.

Thirty-three Cheviot sheep of New Zealand origin with ARQ/ARQ genotypes were part of a study to investigate the effects of additional ovine PrP gene polymorphisms. DNA from sheep-blood samples was isolated and the PrP open reading frame was amplified by PCR as described previously (Goldmann et al., 2005), with the addition that the full ORF was sequenced from PCR products amplified with oligonucleotides MP52d (5'-CTTACGTGGGCATTGGATGC) and MP16u (5'-AACAGGAAGGTTGCCCTATCC). Automated sequencing on an ABI Prism 377 sequencer was performed with a BigDye Terminator kit according to the manufacturer’s guidelines (Applied Biosystems). The available ORF sequences were read to confirm standard genotypes (codons 136, 154 and 171) and were scanned for additional polymorphisms between codons 110 and 243. Seven sheep revealed a C to T transition in the second position of codon 168, leading to an amino acid substitution from proline (P) to leucine (L). The L168 allele encoded A136, L141, R154, Q171 and was found on a haplotype with two silent mutations at codons 231 (c) and 237 (g), both of which have been described previously (Westaway et al., 1994). The DNA polymorphism creates a restriction fragment-length polymorphism motif on the ARL168Q haplotype for the restriction enzyme Spe1.

Several independent experiments using BSE challenges of Cheviot sheep are ongoing at the IAH and have resulted in BSE-affected animals and in healthy survivors that have remained disease-free for significantly longer post-inoculation than the affected animals.

In the first experiment, one ARQ/ARQ and nine ARQ/AHQ sheep were inoculated with 0·2 g BSE-affected cattle-brain homogenate by the intravenous (i.v.) route. These sheep have been reported before by Hunter et al. (2002) and were the positive controls for a blood-transfusion study. Two out of the ten animals had developed BSE at that time. We can now report that a total of eight animals have shown clinical signs of BSE and they were culled according to UK Home Office regulations, with a mean IP of 702 days (SD, 61 days) post-inoculation. All animals were confirmed as BSE-positive by detection of brain vacuolation and detection of PrPSc by immunocytochemistry or immunoblotting (data not shown). PrP genotyping revealed heterozygous genotypes at codon 141 (see below), but not at codon 168, for which they were all P168/P168. Two out of the ten animals challenged i.v. with BSE remain healthy at the time of writing, both 2150 days post-inoculation and 1365 days after the longest IP recorded in the eight affected animals. The two survivors had the genotype P168/L168. This survival period is highly significant and appears to be associated with the presence of the leucine substitution in codon 168. As the two surviving sheep are still alive, we cannot tell at the moment whether the resistance is complete (that they will survive for a normal lifespan without TSE signs) or partial (that they will eventually succumb to the experimental BSE). A study of preclinical BSE in sheep at the IAH demonstrated, surprisingly, no infectivity or pathological PrP accumulation in their tonsils, despite a prominent infection of other lymphoid tissues, suggesting that pathological PrP detection in tonsil biopsies is not a reliable diagnostic test for early BSE in sheep (M. E. Bruce, personal communication). Consequently, to avoid false-negative results, tonsils were not tested. The animals will be kept under surveillance for clinical signs of disease.

In a different BSE experiment, 24 ARQ/ARQ sheep were transfused with whole blood (WB) or buffy-coat preparations (BC) from BSE-inoculated donor sheep (Houston et al., 2000; Hunter et al., 2002). Five transfusions were performed with blood from clinically positive donors. Three P168/P168 homozygous animals developed clinical BSE at 560 days (SD, 32 days) and two P168/L168 heterozygotes have survived (and are still alive at the time of writing) without clinical disease for post-transfusion periods of 1475 and 1980 days. One survivor was challenged by transfusion of BC and the other with WB. Both types of inoculum have been shown to produce similar IPs when susceptible animals were challenged, i.e. WB and BC from the same donor animal resulted in IPs of 556 and 531 days, respectively.

The other transfusions were conducted with material from inoculated animals that had not developed clinical disease when the blood was taken. Two of these recipients died of BSE after IPs of 536 and 610 days. A third animal was culled at 1139 days after transfusion without showing clinical signs, but was found to be BSE-positive for PrPSc. All three sheep were P168/P168. Eleven recipient survivors (alive at time of writing) are of the P168/P168 (10) and P168/L168 (one) genotypes, with the shortest post-transfusion period of 1500 days and the longest of 2620 days (Table 1). It is very likely that survival in some of these sheep is due to the fact that the pre-clinical donors were not BSE-affected.

Survival of a TSE inoculation is normally only observed for reasons of genetic resistance to disease, low effective titre of the agent or inefficiency of the route of infection. The i.v. route has rarely been used in sheep TSE challenges before, but our experiments have shown that it can be an effective route of infection. It can also be inferred from the IPs that the effective titre in these experiments is similar to that of other BSE sources. The absence of the ARL168Q allele in any of the affected animals and the survival of P168/L168 heterozygotes therefore lead to the conclusion that the L168 allele is associated with the resistance of sheep to BSE challenge by either the i.v. inoculation or i.v. transfusion route (Tables 1 and 2).

To test whether the L141F polymorphism was associated in these challenges with the susceptibility to BSE, we also genotyped codon 141 in these animals. Of the 14 affected
animals challenged by i.v. inoculation or i.v. transfusion, six were genotype F 141/F141; the other eight were L 141/F141. The current survivors could be divided into nine homozygous F 141/F141, seven heterozygous L 141/F141 and one homozygous L141/L141 sheep. BSE susceptibility for the i.v. route does therefore not associate with the codon 141 polymorphism.

Although BSE susceptibility is not controlled by codon 141, it is still possible that the IP is modified by this polymorphism. Our analysis showed that the mean IP of four F 141/F141 Cheviot sheep was 572 days (SD, 34 days) after i.v. or i.v. transfusion challenge from clinically and pathologically confirmed donors. The mean IP of five homozygous F141/F141 Cheviot sheep challenged i.c. with 0.05 g BSE cattle-brain homogenate was 608 days (SD, 38 days). Neither mean IP is significantly different from the mean IP of 537 days (SD, 33 days) that resulted after the same inoculation regime was applied to 12 homozygous L141/L141 sheep of the Cheviot, Suffolk and Poll Dorset breeds (Table 1). There are only limited data available for L141/F141 heterozygous sheep and no conclusion can be drawn yet with regard to the heterozygous genotype.

The association of two adjacent codon polymorphisms (168L and 171R) with decreased susceptibility to TSE suggests importance of this structure for the underlying disease mechanisms. PrP protein-structure studies show that both positions are close to or part of a loop between helix 1 and helix 2, comprising codons 169–177. This loop appears quite disordered in most PrP proteins, but the spatial arrangement can be changed considerably by its amino acid sequence (Gossert et al., 2005). It is intriguing to speculate that the substitution of proline with hydrophobic leucine changes the conformation of the loop so that interactions with other proteins or PrP dimerization are

Table 1. Summary of PrP genotypes, incubation periods and survival times for BSE challenges by various routes of inoculation

<table>
<thead>
<tr>
<th>Genotype (136, 141, 154, 168, 171)</th>
<th>n</th>
<th>Route*</th>
<th>Incubation period (days) (SD)</th>
<th>Survival period (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFRPQ/AFRPQ</td>
<td>1</td>
<td>i.v.</td>
<td>605</td>
<td></td>
</tr>
<tr>
<td>AFRPQ/ALHPQ</td>
<td>7</td>
<td>i.v.</td>
<td>716 (50)</td>
<td></td>
</tr>
<tr>
<td>AFRPQ/ALRLQ</td>
<td>2</td>
<td>i.v.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AFRPQ/AFRPQ</td>
<td>3</td>
<td>i.v.-TRANS-C</td>
<td>560 (32)</td>
<td></td>
</tr>
<tr>
<td>AFRPQ/ALRLQ</td>
<td>2</td>
<td>i.v.-TRANS-C</td>
<td>1475†, 1980†</td>
<td></td>
</tr>
<tr>
<td>AFRPQ/AFRPQ</td>
<td>2</td>
<td>i.v.-TRANS</td>
<td>536, 610</td>
<td></td>
</tr>
<tr>
<td>ALRPQ/AFRPQ</td>
<td>1</td>
<td>i.v.-TRANS</td>
<td>1339‡</td>
<td></td>
</tr>
<tr>
<td>AFRPQ/AFRPQ</td>
<td>9</td>
<td>i.v.-TRANS</td>
<td>1500–2620†</td>
<td></td>
</tr>
<tr>
<td>AFRPQ/ALRLQ</td>
<td>1</td>
<td>i.v.-TRANS</td>
<td>2200†</td>
<td></td>
</tr>
<tr>
<td>ALRPQ/ALRPQ</td>
<td>1</td>
<td>i.v.-TRANS</td>
<td>2620†</td>
<td></td>
</tr>
<tr>
<td>AFRPQ/AFRPQ</td>
<td>1</td>
<td>i.v.-TRANS</td>
<td>2239</td>
<td></td>
</tr>
<tr>
<td>ALRPQ/ALRPQ</td>
<td>1</td>
<td>i.v.-TRANS</td>
<td>1444</td>
<td></td>
</tr>
<tr>
<td>AFRPQ/AFRPQ</td>
<td>5</td>
<td>i.c.</td>
<td>608 (38)</td>
<td></td>
</tr>
<tr>
<td>ALRPQ/ALRPQ</td>
<td>12</td>
<td>i.c.</td>
<td>537 (33)</td>
<td></td>
</tr>
</tbody>
</table>

*i.v., Intravenous; i.v.-TRANS-C, intravenous blood transfusion from clinically positive donors; i.v.-TRANS, intravenous blood transfusion from infected, but not clinically positive donors; i.c., intracerebral.

†Animals are still alive at time of writing.
‡This animal suffered an intercurrent death and showed PrPSc deposition; the survival time is therefore not the incubation period defined by occurrence of clinical BSE signs.

<table>
<thead>
<tr>
<th>Allele (n)</th>
<th>Susceptible* (%)</th>
<th>Resistant† (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFRPQ (45)</td>
<td>42-2</td>
<td>57-8</td>
</tr>
<tr>
<td>ALRPQ (3)</td>
<td>33-3</td>
<td>66-7</td>
</tr>
<tr>
<td>ALRLQ (5)</td>
<td>0</td>
<td>100‡</td>
</tr>
<tr>
<td>ALHPQ§ (7)</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

*Animals had incubation periods of ≤1400 days and showed PrPSc accumulation.
†Animals are still alive >1400 days after inoculation.
‡x² = 5, P < 0.05.
§The distribution of this allele was influenced by non-random selection of sheep for the two challenge experiments.

Table 2. Association between PrP alleles and susceptibility/resistance to BSE after intravenous inoculation

See Table 1 for allele description.

<table>
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<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

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inhibited, with possible consequences for PrP<sup>C</sup> to PrP<sup>Sc</sup> conversion. Indeed, in vitro conversion experiments have shown very low conversion of the ARL<sup>168Q</sup> allele with BSE or scrapie inoculum (Kirby et al., 2006). This PrP codon 168 polymorphism is not unique. For caprine PrP, a P168Q polymorphism has been described (Billinis et al., 2002) and our study of European red deer PrP sequences has revealed another variant in this position, P168S (M. Perucchini & W. Goldmann, unpublished observations). In vitro conversion assays are under way to investigate these amino acid changes for their PrP<sup>Sc</sup> conversion efficiency.

Scrapie cases with the ARL<sup>168Q</sup> allele have not been detected in scrapie-affected flocks in the UK (W. Goldmann, unpublished observations). An allele frequency of around 1.4% in scrapie-affected flocks (Goldmann et al., 2005) will make it difficult to establish association with scrapie resistance from flock studies and may require experimental inoculation with classical and atypical scrapie. Scrapie has been reported in goats with heterozygous ARQ/ARQ<sup>168Q</sup> genotypes (Papasavva-Stylianou et al., 2005; Acutis et al., 2006), which may indicate that the type of amino acid change in this position plays an important role. There are obviously no data for European red deer, as they do not have TSE disease, but it would be of great interest to investigate the association of the cervid P168S polymorphism with chronic wasting disease by in vitro or in vivo assays.

Recently, it was reported that Nor98 and other atypical scrapie cases showed a significant association of the AF<sup>141RQ</sup> allele with susceptibility (Moum et al., 2005). In the presented experiments, there was no indication of a codon 141 association with BSE susceptibility and, equally, we have found no association with SSBP/1 scrapie susceptibility (W. Goldmann, unpublished results). This is another example of the specificity regarding the association of PrP genotype and TSE agent strain and implies that it is premature to infer from our BSE challenges the influence that the ARL<sup>168Q</sup> allele may exert on the variety of classical and atypical scrapie strains in sheep.

Many different PrP genotyping methods are applied worldwide and it is important to consider that some of them may fail for certain samples, due to the closeness of the codon 168 and 171 polymorphisms. For example, methods using oligonucleotides upstream of codon 171 have to take into account the fact that the codon 168 polymorphism is only nine bases upstream of the 171 Q/R polymorphism. Therefore, it is likely to interfere with optimal primer binding. Methods may therefore have to be adjusted and caution should be applied when genotyping uncharacterized breeds or other species such as deer and goats, as similar problems may occur.

Since the discovery of the protective effect of the R171 allele in sheep BSE (Goldmann et al., 1994), this study is the first to reveal a new ovine polymorphism that exhibits significant protection against challenge with the BSE agent. Future investigations of natural scrapie will establish whether its effect is equally significant for natural scrapie outbreaks.

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**References**


