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Citation for published version:

Digital Object Identifier (DOI):
10.1128/jvi.00448-11

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Peer reviewed version

Published in:
Journal of Virology

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Proteinase K-Resistant Material in ARR/VRQ Sheep Brain Affected with Classical Scrapie Is Composed Mainly of VRQ Prion Protein\textsuperscript{\dag}\textsuperscript{†}

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Received 5 March 2011/Accepted 24 August 2011

Classical scrapie is a prion disease in sheep and goats. In sheep, susceptibility to disease is genetically influenced by single amino acid substitutions. Genetic breeding programs aimed at enrichment of arginine-171 (171R) prion protein (PrP), the so-called ARR allele, in the sheep population have been demonstrated to be effective in reducing the occurrence of classical scrapie in the field. Understanding the molecular basis for this reduced prevalence would serve the assessment of ARR adaptation. The prion formation mechanism and conversion of PrPC to the scrapie-associated form (PrP\textsuperscript{res}) could play a key role in this process. Therefore, we investigated whether the ARR allele substantially contributes to scrapie formation in naturally infected heterozygous 171Q/R animals. Two methods were applied to brain tissue of 171Q/R heterozygous sheep with natural scrapie to determine the relative amount of the 171R PrP fraction in PrP\textsuperscript{res}, the proteinase K-resistant PrP\textsuperscript{Sc} core. An antibody test differentiating between 171Q and 171R PrP fragments showed that PrP\textsuperscript{res} was mostly composed of the 171Q allelotype. Furthermore, using a novel tool for prion research, endoproteinase Lys-C-digested PrP\textsuperscript{res} yielded substantial amounts of a nonglycosylated and a glycosylated PrP\textsuperscript{res} fragment. These results support a nearly zero contribution of 171R PrP in PrP\textsuperscript{res} of 171R/Q field scrapie-infected animals. This is suggestive of a poor adaptation of classical scrapie to this resistance allele under these natural conditions.

Transmissible spongiform encephalopathies (TSEs) or prion diseases are infectious neurological diseases for which susceptibility and transmissibility are at least dependent on the strain of the agent and the prion protein (PrP) genotype of the host, while other host factors also play a role (3, 6, 13, 18). The archetypal example is natural scrapie in sheep, for which the infectious nature was first shown by Cuillé and Chelle following experimental infection of goat and sheep (15). In humans, infectious nature was first shown by Cuillé and Chelle following experimental infection of goat and sheep (15). In humans, transmissible spongiform encephalopathies (TSEs) or prion diseases are infectious neurological diseases for which susceptibility and transmissibility are at least dependent on the strain of the agent and the prion protein (PrP) genotype of the host, while other host factors also play a role (3, 6, 13, 18). The archetypal example is natural scrapie in sheep, for which the infectious nature was first shown by Cuillé and Chelle following experimental infection of goat and sheep (15). In humans, TSEs have been identified and found to be dependent on both prion strain and PrP polymorphisms. For classical scrapie and bovine spongiform encephalopathy (BSE) in sheep, three important amino acid polymorphisms that influence susceptibility and transmission have been described, i.e., alanine (A) to V at codon 136, arginine (R) to histidine (H) at codon 154, and glutamine (Q) to R at codon 171 (3, 28, 29, 57). In atypical/Nor98 scrapie, a form of scrapie that has poor transmission properties, susceptibility mainly correlates to a substitution of R to H at codon 154 or leucine (L) to phenylalanine (F) at codon 141 (19, 43, 53). Taking the major TSE transmission-
related polymorphisms of sheep into account, a 3-amino-acid nomenclature for codons 136, 154, and 171 is used, and $\text{A}_{171}\text{R}_{154}\text{Q}_{171}$ (usually indicated ARQ) is considered to be the wild-type allele. For classical scrapie forms in sheep, the levels of susceptibility in the context of amino acid substitutions have been ranked in the following order: VQK, ARQ, AHOQ, and ARR. Such information has led to successful scrapie eradication programs in different European countries by use of a genetic breeding strategy targeted to the enrichment of the 171R allele (23, 40, 62).

A concern of such breeding strategies is whether this type of genetic selection might lead to the emergence or adaptation of a new TSE strain that would replicate more efficiently using the R171 allele. However, for classical scrapie, such a condition has hardly been reported. It is known that the 171R allele historically occurs in many breeds at relatively high frequencies, though there is little evidence of scrapie in sheep carrying this allele. For example, only a small number of scrapie cases have been associated with ARR/VRQ heterozygous sheep, while scrapie outbreaks in ARQ/ARR sheep with scrapie are very rare, and only three natural cases in sheep that are 171R homozygous have been reported (14, 22, 30, 33). Of significant importance is whether or not scrapie-positive heterozygous ARR/VRQ sheep carry equimolar amounts of both PrP alleles in the PrP$^{\text{Sc}}$ fraction and if this might be indicative for a tendency that such cases will lead to enhanced scrapie transmissibility within the 171R-carrying sheep population. Thus far, in the one single ARR/VRQ scrapie field case studied, no PK-resistant ARR material was detected (41). In vitro studies already indicate that the binding behaviors of the different PrP allelic forms to PrP$^{\text{Sc}}$ are comparable on the molecular level of PrP (51), while conversion studies have shown that the 171R allele has a relatively low tendency to become PK resistant (9, 10). After oral BSE challenge of 1- to 2-week-old ARR/ARR lambs, appreciable levels of ARR PrP$^{\text{Sc}}$ and PrP$^{\text{Res}}$ were detected only in the spleen of one out of three asymptomatic animals euthanized at 10 months after infection (2).

In this study, we determined the involvement of ARR PrP (further referred to as 171R) in PrP$^{\text{Res}}$ relative to wild-type 171Q PrP (further referred to as 171Q) in a group of 8 naturally infected sheep with ARR/VRQ scrapie. We used both an immunohistochemical discrimination method and a biochemical separation technique. For the first method, the binding of monoclonal antibody (MAb) SAF84 to PrP was highly dependent on 171Q-containing allelotypes. The second approach was a novel technique for the quantitative generation of a 6-kDa PrP fragment from codons 114 to 188 (114-188PrP) using endoprotease Lys-C digestion of PrP$^{\text{Res}}$. Generating this PrP$^{\text{Res}}$ fragment has been associated with 171Q-containing allelotypes to PrP codons 114 to 188 (114-188PrP) using endoprotease Lys-C digestion of PrP$^{\text{Res}}$. Generating this PrP$^{\text{Res}}$ fragment has been associated with 171Q-containing allelotypes to PrP codons 114 to 188 (114-188PrP). This PrP$^{\text{Res}}$ fragment is resistant to proteinase K digestion (PrP$^{\text{Res}}$) and is associated with 171Q-containing allelotypes to PrP codons 114 to 188 (114-188PrP).

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Tissue treatments. Ten percent (wt/vol) brain stem homogenates were prepared in lysis buffer and digested with PK at 37°C, and PrP$^{\text{Res}}$ was partially purified by precipitation with 1-propanol as described previously (31). For PrP$^{\text{Res}}$ analyses, pellets were directly subjected to SDS-PAGE and Western blotting.

For further biochemical identification of 171Q and 171R allele fragments, pellets containing 10 mg tissue equivalent (TEs) were denatured by suspension in 50 μl of 8 M guanidine hydrochloride containing 40 mM dithiothreitol, and after 5 min at ambient temperature, the protein was precipitated by mixing with 10 volumes of ice-cold (−20°C) methanol, kept for 30 min at −20°C, and then centrifuged at 18,000 × g for 20 min at 4°C (5417R; Eppendorf centrifuge). The final pellet was resuspended in 150 μl of 1% (wt/vol) N-lauroylsarcosine sodium salt in 0.1 M Tris-HCl, pH 8.5, containing 40 mM dithiothreitol, heated for 5 min at 96°C, and cooled down to ambient temperature. Cleavage of protein at the carbonyl-terminus of lysines by Lys-C was performed by adding 15 μl of a stock solution of 0.015 U/μl of enzyme and incubating at 37°C overnight.

1D electrophoresis (SDS-PAGE). Three volumes of Lys-C-digested sample were mixed with 1 volume of NuPAGE® 4% lithium dodecyl sulfate (LDS) sample buffer containing 0.1 M dithiothreitol and denatured by heating at 96°C for 5

**Materials and Methods**

**Chemicals and reagents.** Chemicals were purchased from Merck (Darmstadt, Germany) unless otherwise specified (and were at least analytical reagent grade). All procedures were performed at room temperature unless otherwise stated.
min. After cooling to room temperature, the sample was centrifuged at 16,000 \( \times \) g for 5 min in a Biofuge Pico microcentrifuge. Sample supernatants (10 \( \mu l \) 0.5 mg TE per lane) were then subjected to 1D electrophoresis in 12% bis-Tris NuPAGE gels using morpholineethanesulfonic acid (MES) with antioxidant as running buffer.

**2D electrophoresis.** The protein in the Lys-C-digested sample (165 \( \mu l \)) was precipitated with 10 volumes of ice-cold (\(-20^\circ\)C) methanol (without detectable PrP loss). After consecutive mixing and incubating at \(-20^\circ\)C for 30 min, the sample was centrifuged at 18,000 \( \times \) g (5417R; Eppendorf centrifuge) at 4°C for 20 min. The supernatant was discarded. Further procedures used the supplier’s (Invitrogen) instructions for 2D electrophoresis. The pellet was resuspended and diluted into 165 \( \mu l \) of sample rehydration buffer (SRB2; 90% [vol/vol] ZOOM 2D protein solubilizer 2, 0.6% [vol/vol] ZOOM carrier ampholytes, pH 3 to 10, 0.002% [wt/vol] homophenol blue, 0.5% [vol/vol] 2 M dithiothreitol in water). After centrifugation at 16,000 \( \times \) g for 5 min in a Biofuge Pico microcentrifuge for debris removal, the supernatant was diluted in SRB2 to obtain 2.5 mg TEs in 140 \( \mu l \) per immobilized pH gradient (IPG) strip. ZOOM strips, pH 3 to 10, were rehydrated with the sample for 1 h in an IPG runner cassette. First-dimension isoelectric focusing occurred in the IPG runner system with a stepwise voltage program of from 200 V to 2,000 V for a total run time of 80 min until the dye front reached the anode. The IPG strips were then equilibrated for the second-dimension separation by incubation in reducing and denaturing solution for 15 min at ambient temperature according to the supplier’s protocol, followed by alkylation with iodoacetamide for 15 min. The equilibrated IPG strip was applied to the NuPAGE Novex bis-Tris ZOOM gel. The reference lane was loaded with a mixture of 5 \( \mu l \) SecBlue Plus2 marker and 5 \( \mu l \) of the 1D SDS-PAGE sample prepared in the same way described above for the IPG strip to obtain 0.25 mg TE. Second-dimension electrophoresis was performed for 35 min at 200 V with MES running buffer and antioxidant.

**WB, detection, and quantitation.** The subsequent WB procedures—electrotransfer, immunostaining, and the development of luminescence (using CDP-star as the substrate for alkaline phosphatase)—were performed as described previously (33). Antibodies were used at the concentrations indicated. To obtain digital images, individual films were exposed to the blots for different time periods (i.e., 30 s, 1 min, 3 min, and 6 min) and digitalized using a scanner. Calculations on these digital readings were performed using ImageQuant (version 5.2) software. Additional WB studies on PK resistance of PrP\(^{\res}\) were performed using a triplex WB system, which uses a recently published fluorimetric method which allows immunochemochemical quantification of protein without enzymatic enhancement (31). Using both Western blot systems, the fractions of R and Q PrP\(^{\res}\) present in a typical digest were obtained from the ratios of signals obtained with MAb L42 and SAF84 with L42 binding to both PrP allelotypes and SAF84 binding only to the 171Q PrP allele.

RESULTS

**Discrimination of R and Q at codon 171 in synthetic PrP peptides and recombinant PrP using SAF84 binding.** The presence or absence of the allele 171R expression product in PrP\(^{\res}\) material from heterozygous (171Q/R) sheep was first investigated by an antibody discrimination assay. To this end, the PrP-specific antibody SAF84 was employed since, using epitope mapping with Pepscan analysis, it appeared to bind the PrP-specific antibody SAF84 was employed since, using epitope mapping with Pepscan analysis, it appeared to bind the epitope sequence with 171Q, while there is no binding observed when the polymorphic amino acid R171 is present. SAF84 was applied at 0.05 \( \mu g/ml \) of all 220 15-mers, only peptides 151 to 190 are displayed. All other overlapping peptides (data not shown) of the 256 residues of the ovine PrP sequence (21) exhibited background signals similar to those shown in panel b.

**Estimation of 171R/Q-containing allelotype in PrPres from sheep brain tissue using SAF84.** PrP\(^{\res}\) was prepared from brain homogenates of scrapie-positive sheep with the ARR/VRQ, VRQ/VRQ, or ARQ/VRQ genotype. From results with recPrP, it was assumed that 171Q-dependent SAF84 binding to PrP\(^{\res}\) would be less strong if 171R PrP\(^{\res}\) was present in the brain homogenates than if only 171Q PrP\(^{\res}\) was present (compared to a non-polymorphism-dependent MAb like L42). Using 1D SDS-PAGE followed by Western blotting with MAb SAF84 and L42 in parallel, the SAF84/L42 ratios were slightly lower in the 171R/Q heterozygotes than the 171Q/Q homozygotes (0.95 ± 0.08 and 0.99 ± 0.08, respectively).

FIG. 1. Pepscan analysis of antibody SAF84 on overlapping solid-phase 15-mer peptides of two ovine PrP sequences differing only at codon 171. (a) Analysis of 171Q PrP; (b) analysis of 171R allelic variants. With wild-type ovine PrP, this gives 166 YRPVDQY172 as the SAF84 core epitope sequence with 171Q, while there is no binding observed when the polymorphic amino acid R171 is present. SAF84 was applied at 0.05 \( \mu g/ml \). Of all 220 15-mers, only peptides 151 to 190 are displayed. All other overlapping peptides (data not shown) of the 256 residues of the ovine PrP sequence (21) exhibited background signals similar to those shown in panel b.
whereby the differences were not statistically significant (Fig. 2b). If there had been more than 10% of the 171R product present, the ratio would have been 0.89 (using the 0.99 ratio in the 171Q/Q sheep as a 100% 171Q reference). Although the observed ratios were considered equal, if there was any 171R PrPres present in the brain of 171R/Q heterozygote sheep, it was calculated that the 171R PrP relative level constituted 4% of the total PrP res material on the basis of these ratio differences.

Design of a biochemical approach for identification of 171R and 171Q PrPres in scrapie material. Since the Q-to-R amino acid change at position 171 involves an obvious charge inversion, we attempted to find potential cleavage fragments of PrPres that could be measured by 2D analysis. We rationalized, in principle, that following treatment with the enzyme endoproteinase Lys-C (which cleaves after lysine [K] residues), a 75-amino-acid-residue PrP fragment spanning codons 114 to 234. The lysine residues which are cleaved following treatment with Lys-C are indicated by arrows. The result PrP fragment was calculated to have an approximate molecular mass of 8 kDa and an isoelectric point (pI) of between 7 and 8 (34). It was also postulated that the PrP fragment, when derived from wild-type 171Q PrP, would have a net ionic charge of 0, whereas when derived from 171R PrP, it would carry a net ionic positive charge of 1 (Fig. 3). Thus, the 171R-containing fragment would have a calculated pI of between 8 and 9 and the two allelic products would differ by about 1 pI unit and could be readily distinguished using 2D electrophoresis.

Generation of a 144-188PrP fragment from scrapie brains. PrPres from scrapie tissue samples was digested with Lys-C and then resolved by 1D electrophoresis. Subsequent analysis using Western blotting with antibodies L42 and SAF84 showed the disappearance of the typical three protein bands corresponding to PrPres and the emergence of smaller polypeptide species migrating at 6 kDa and at 14 kDa (Fig. 4). These were presumed to be the nonglycosylated and monoglycosylated forms, respectively, of the generated 114-188PrP fragment containing the epitopes of MAbs L42 and SAF84. The epitopes of antibodies 12B2, 9A2, and FH6 were removed, as expected (Fig. 3b).
The 2-kDa discrepancy between a nonglycosylated 6-kDa product (shown in Fig. 4) and the 8-kDa product that we theorized would result from this approach is likely a consequence of the imprecision of the technique for estimating the molecular masses of proteins with molecular masses below 12 kDa and where the charges of amino acid side chains influence migration behavior. Unexpectedly, however, the N-terminal domain of the PrP fragment, which we anticipated to contain the epitope of the MAb 6C2 (amino acids 114 to 120), was not present, as illustrated by failure of this antibody to bind the PrP fragment (Fig. 5a). Since Lys-C cleavage leads to a free amino terminus, it is conceivable that antibody binding is dependent on an intact peptide bond at residue 114, the first amino acid of the 6C2 epitope. Indeed, in a blocking ELISA, the binding of 6C2 to coated ovine PrP could be prevented by peptides only if the entire peptide sequence from codons 114 to 120 was present with an intact peptide bond but not when the 114 residue was terminally present with a free amino terminus (Fig. 5b). This observation, in conjunction with the binding of MAb L42 and SAF84 but not 12B2, 9A2, and FH6 and the resultant 6-kDa fragment, collectively indicated that the postulated cleavage by Lys-C at residues 113K and 188K had occurred to completion (as opposed to the variable levels of cleavage that could have occurred at lysines at positions 25, 26, 29, 104, 107, 109, 197, and 207 within PrP).
Identification of 171Q- and 171R-containing PrPres fragments after 2D electrophoretic separation. The 114-188PrP fragment generated by Lys-C treatment of recombinant ovine 171R- and 171Q-containing PrP variants was further characterized using 2D electrophoresis and Western blotting (with MAb L42). Figure 6a shows that the major cleavage products from the Lys-C digest migrated as expected, whereby pIs of approximately 8 for the 171R recPrP-derived polypeptide and 7 for the 171Q-containing recPrP polypeptide were observed. Also, in 171R recPrP, the pI 8 protein spot showed a positive reactivity against MAb L42, which was absent when the 171Q-specific MAb SAF84 was used. This confirmed that the pI 8 spot consisted of 171R protein.

This assay was repeated using PrPres prepared from homozygous 171Q/Q (VRQ/VRQ or ARQ/ARQ) sheep. The digestion products resolved at pIs of 7 and lower for both the nonglycosylated (6 kDa) and the glycosylated 114-188PrP fragment (14 kDa), but none did so above this pI value (Fig. 6b). Yet, in five out of eight codon 171 heterozygous (all ARR/VRQ) cases, 171R 114-188PrP fragment material was marginally evident at pI 8 only at the nonglycosylated 6-kDa migration position (Fig. 6b, spot above the arrow). This suggests that 171R-containing polypeptide material is of a lower concentration at pI 8, while nonglycosylated and glycosylated 171Q materials appeared to be major spots at pI 7 and lower. The pI 8 spot was not detectable in the remainder of scrapie-infected 171Q animals investigated, nor was it detectable in any 171Q/Q animals (Table 1). In the five cases where a 171R protein spot was observed, the amount of ARR material in the pI 8 spot approximated, on average, 2.6% (range, 0 to 8.1%) of the total protein spots at the glycosylated and nonglycosylated positions (ARR plus VRQ spots, following densitometric analysis of digitalized films). These five 171Q/R heterozygous cases having the pI 8 spot were by 1D analysis among the strongest positive PrPres cases. In summary, the 171R/Q heterozygous scrapie-infected sheep investigated did contain the 171R PrP in their PK-resistant PrP Sc material, though at variable concentrations and levels below 9% of total PrPres.

Estimation of proteinase K susceptibility of 171R PrPres. The differential susceptibility of 171R PrPres to PK treatment

<table>
<thead>
<tr>
<th>Case code</th>
<th>Condition at death</th>
<th>Age (mo)</th>
<th>Genotype</th>
<th>IHC result</th>
<th>ARR spot (%)</th>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Tonsil</td>
<td>Brain IHC</td>
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<td>Fallen stock</td>
<td>Unk</td>
<td>VRQ/VRQ</td>
<td>Pos</td>
<td>Pos</td>
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<tr>
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<td>VRQ/VRQ</td>
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<td>Pos</td>
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<td>621553–4035</td>
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<td>573862</td>
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<td>Pos</td>
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<td>ARR/VRQ</td>
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<td>67.2</td>
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<td>Neg</td>
<td>Pos</td>
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<td>547189–4333</td>
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<td>ARR/VRQ</td>
<td>Neg</td>
<td>Pos</td>
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<td>Slaughter</td>
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<td>ARR/VRQ</td>
<td>Neg</td>
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<tr>
<td>2007–20</td>
<td>Slaughter</td>
<td>Unk</td>
<td>ARR/VRQ</td>
<td>Wk pos</td>
<td>Pos</td>
</tr>
</tbody>
</table>

* Cases were either from active surveillance, i.e., those starting with a year number; passive surveillance, i.e., those with a single case number (case 573862); or CVI institutional flock with circulating natural scrapie (i.e., those with a double case number). Unk, unknown; Pos, positive; Neg, negative; Wk pos, weakly positive; ND, not determined.

Polypeptide material in spots at pI 8 was calculated as the percentage of all spots at the 6-kDa and 14-kDa positions, as explained in the Materials and Methods section. ND, not detected.
was investigated by applying enzyme concentrations of from 10 to 250 μg/ml. In this experiment, not only were field scrapie-derived materials used, but also materials derived from experimental animals inoculated intracerebrally with scrapie and BSE. It appeared that in all cases the 171R material was equally resistant to the protease at concentrations between 10 and 50 μg PK/ml and by use of typical experimental conditions (Fig. 7), notwithstanding 1- to 100-fold variations in PrP<sup>res</sup> level between individual samples (see Fig. S1 in the supplemental material). For PK concentrations of from 50 to 250 μg PK/ml, there was a 25 to 50% reduction in PrP<sup>res</sup> signal in the R allele-containing samples but not in the homozygous 171Q/Q cases of scrapie and BSE. Interestingly, when brain material from 171R/Q heterozygous sheep experimentally infected with BSE was digested under standard PK conditions of 50 μg PK/ml, the amount of 171R-containing PrP<sup>res</sup> (on the basis of antibody binding ratios between SAF84 and L42) was about 64% (standard deviation [SD], 10%). This was much higher than that for ARR/VRQ field case material from sheep with classical scrapie, where the 171R-containing fraction represented less than 9% (Fig. S2 in the supplemental material).

**DISCUSSION**

An altered form of prion protein is believed to be the major, if not sole, protein component of the infectious agent in TSE diseases, and expression of the normal isoform in the host is essential for disease transmission. Being so critically involved in disease development, PrP polymorphic sequence variants genetically determine transmissibility properties, such as the species barrier, lymphotropism, and within-species susceptibility, by modulating the interconvertibility of PrP itself. In the study described in this paper, evidence was found for the presence of the 171R protein in PrP<sup>res</sup> from heterozygous ARR/VRQ scrapie sheep, which usually incubate the disease over 6 years under natural conditions and which carry PrP<sup>C</sup> of both the 136V allele, associated with high susceptibility, and the 171R allele, associated with resistance. However, the level of the 171R protein in PrP<sup>res</sup> of heterozygous sheep is very low (if present at all), and in this study, it was quantified to constitute a maximum of 8.1% of the total PrP<sup>res</sup> material in such sheep, contrasting with an expected value of 50% if the expression level and conversion of both allelic products had been equal in the tissue of origin. These data were deduced using two independent methods: a discriminatory antibody tool and a novel procedure to generate and separate 114-188PrP fragments from PrP<sup>res</sup> containing either the 171Q or 171R codon.

First, the use of MAbs SAF84 was chosen because of its selective affinity to bind ovine PrP containing 171Q, in contrast to a lack of reactivity for PrP containing the polymorphic amino acid, 171R. The estimation of the relative amount of the 171R allele in scrapie material obtained using SAF84 was possible by comparison to that obtained using the nondiscriminative antibody L42, which binds both allelic expression products. The approach of using a polymorphism-dependent PrP-specific antibody has proven to be a useful way of determining the genotype status at the codon 171 position in serum of sheep and for confirming the presence of the 171R allele in PrP<sup>res</sup> in spleen and brain of 171R/R homozygous sheep experimentally challenged with bovine BSE (8). These kinds of estimations can be safely carried out since the PRNP gene that encodes PrP is likely expressed equally at the mRNA level for different genotypes and different cells, even though PrP levels do vary depending on the tissue (8, 17, 36, 41, 54). Thus, it is assumed that the different alleles of PrP<sup>C</sup> studied are present in equal amounts in brains of healthy heterozygous sheep, yet in heterozygous ARR/VRQ scrapie-infected individuals, the relative contributions of the various levels of allele expression products in the PrP<sup>res</sup> material are not equal.

The second procedure that we employed is a new tool to
generate a polypeptide core fragment from codons 114 to 188 of ovine PrP from PrP\textsuperscript{res} by using endoproteinase Lys-C digestion. This allowed a clear-cut discrimination of both codon 171 allotypic PrP forms (R/Q) in 2D electrophoresis on the basis of charge differences. This enzyme has been used before in a different context for studying the C-terminal fragment carrying a glycosylphosphatidylinositol anchor (58). In principle, Lys-C would release the 114-188PrP fragment from PrPs of nearly all animal species, including humans, the exceptions being some animal species where the C-terminal lysine for cleavage would be expected to occur at PrP codon 167K (mink) or 197K (felines) (1, 35, 65). In recombinant PrP as well as proteinase K-treated scrapie PrP\textsuperscript{res}, the 114-188PrP polypeptide fragment generation method appeared to be highly selective and sufficiently robust for the discrimination of 171R/Q-containing ovine PrP variants under the experimental conditions described. This was evidenced in the 1D Western blotting analyses, whereby the nonglycosylated and monoglycosylated PrP fragment forms were selectively detected using various PrP-specific antibodies; i.e., the binding of MAb 6C2 appeared to be absent if the epitope N terminus was processed into a free amino terminus (as was seen to occur after lysine 113).

Both the selective antibody approach using MAB SAF84/L42 ratios and the isolation of the 171R fragment by 2D electrophoresis demonstrated that only marginal amounts of the 171R-containing PrP were present (if at all) and constituted less than 9% of total PrP\textsuperscript{res}. The differential migration at pI 7 and pI 8 of the 6-kDa PrP fragment generated after Lys-C digestion when using 171Q and 171R recPrP, respectively, was a consistent indicator that equivalent PrP\textsuperscript{res} fragments were generated from scrapie brain. We concluded that the involvement of the 171R PrP allele product in PrP\textsuperscript{res} formation is limited in classical scrapie field cases in ARR/VQ sheep. The nature of the additional protein spots at a pI of <7 that were observed in both recPrP samples and sheep PrP\textsuperscript{res} preparations is, as yet, unknown and requires further investigation. Unlike other studies, incomplete processing by PK seems to be an unlikely explanation since the migration differences in the second dimension are very small and the ablation of antibody binding by MAB 6C2 indicates that cleavage at lysine 113 was efficient. Therefore, it is conceivable that posttranslational differences between the additional protein spots are the more likely explanation. For example, methionine oxidation or methylated arginines could result in an increase of more acidic methionine residues or a decrease in the number of basic arginine residues, which would subsequently affect the overall net charge and thus explain the differently charged spots at a pI of <7 (12, 49).

The limited involvement of resistance allele 171R PrP in PrP\textsuperscript{Sc} formed during scrapie infection corroborates the findings observed in \textit{in vitro} conversion studies with cellular and recombinant ovine PrPs when sheep are exposed to scrapie or BSE, in addition to data generated by mass spectrometric study of a single scrapie-infected ARR/VQ sheep (9, 10, 32, 41). Our findings that 171R in PrP\textsuperscript{res} was detected in limited amounts and in only 5 out of 8 ARR/VQ sheep naturally infected with classical scrapie indicate that the 171R PrP\textsuperscript{res} is not always present in PK-resistant material. Alternatively, it may be present, but at levels less than our current assay limits will permit detection.

A selective conversion of susceptibility-related 171Q PrP\textsuperscript{C} to PrP\textsuperscript{Sc} in classical sheep scrapie might occur when in heterozygotes a highly susceptible allele, such as a codon 136V PrP, and a highly resistant allele, such as 171R PrP, are present, as was suggested before on the basis of \textit{in vitro} conversion assays with sheep PrP (9). Similarly, in human heterozygous M/V129 carriers with GSS having either the susceptibility mutant codon 198 (Phe \rightarrow Ser) or codon 217 (Gln \rightarrow Arg), this mutant PrP appeared to be selectively involved in amyloid formation (59). Other studies on the inherited codon 102L mutant in human GSS patients (P102L heterozygous) revealed a variable but minor presence of wild-type PrP of up to 10% of total PrP\textsuperscript{res} using immunochemical techniques with codon 102-insensitive and -sensitive PrP-specific antibodies, similar to the method used here with MABs L42 and SAF84, respectively (66). However, in patients either with familial CJD and heterozygous for the inherited prion disease-related PrP codon 210V/I or with sporadic CJD with codon 129 M/V, both alleles were equally present in the proteinase K-resistant prion fraction (55). Our analysis of experimental BSE samples from sheep with the ARR/ARQ allele containing up to 65% 171R PrP in PrP\textsuperscript{res} might indicate either that in this combination both ARQ (wild-type) and the resistant ARR alleles convert equally well or that BSE behaves differently from classical scrapie. A difference in allele composition in PrP\textsuperscript{res} of heterozygotes between infections in the field and experimental infections might also play a role, though such information is lacking for sheep. Furthermore, heterozygosity can be a protective factor by itself, as was shown in cell culture (46).

It seems unlikely that the low/absent levels of the 171R-containing PrP in PrP\textsuperscript{res} from ARR/VQ sheep with scrapie result from the fact that while the 171R allele is converted to PrP\textsuperscript{Sc}, it is more susceptible to proteinase K and therefore would not be detected. The analysis of samples from a single ARR/ARR sheep with classical scrapie or sheep with BSE (3 cases studied) did not show differences in susceptibility of PrP\textsuperscript{res} to proteolytic digestion at the PK concentrations (50 \mu g PK/ml) used for our studies. At higher concentrations of PK, however, PrP\textsuperscript{res} 171R allele carriers appeared to be more PK susceptible than the 171Q/Q homozygotes. In two 171R/R field cases with classical scrapie, PrP\textsuperscript{res} was shown to be more protease susceptible than the 171Q/Q homozygous scrapie individuals, where higher PK concentrations, at and above 50 \mu g PK/ml, were used (22). A difference in the methodology used must also be considered, since we used Western blotting for PrP\textsuperscript{res} detection, which revealed the whole remaining part of the PrP molecule present in PK-digested PrP\textsuperscript{Sc}, while the study referred to above used an ELISA with octarepeat (56-88PrP)-specific antibody for PrP\textsuperscript{res} capture. The octarepeat region might be more prone to removal by PK than the large region at the C terminus of the octarepeats. Indeed, under our conditions, such octarepeats can be considered largely removed in both classical scrapie and BSE PrP\textsuperscript{res} from sheep, while the part of PrP at the C terminus of these repeats remains available for detection (60). Finally, the possibility remains that under certain conditions the infectious entity associated with PrP conformations may be protease sensitive.

In the study of Rigter and Bossers, the binding of the 171Q or 171R allele product to PrP\textsuperscript{Sc} appeared to be equal (51). Therefore, it will be important to understand the mechanisms
by which PrPC undergoes conversion to its disease-associated isoform and whether resistance to disease in heterozygotes is a consequence of poor conversion of a resistance allele (in this case, 171R PrP) or whether resistance-associated alleles somehow inhibit the entire process of conversion (including the conversion of alleles associated with susceptibility to infection). For other TSEs, including those caused by new and emerging strains, it is likely that other polymorphisms will play a more significant role in modulating the conversion process.

In conclusion, our results strongly support current genetic models where atypical scrapie does not transmit under field conditions (39). In our study, the methods used were not appropriate to estimate the level of 171R PrP accumulation in atypical scrapie material of 171Q/R heterozygotes since, when using PK, our digestion conditions destroy most of the PrP region around position 171 eventually present.

Our data, showing the limited presence of 171R PrP in the PrPSc fraction of infected heterozygous sheep, could imply that 171R/R sheep infected with classical scrapie have a low risk of developing a new form of prion disease which has specifically adapted for this resistance-associated allele. If there was a significant risk for such adaptation, scrapie or new forms of TSE in sheep carrying this resistance allele would likely occur at frequencies much higher than those actually observed in 171R carriers, given that this allele historically represents more than 30% of the gene pool in most breeds (39). For other strains of prion disease, i.e., atypical/Nor98 scrapie or BSE, it is likely that the contributions of allelic PrP variants other than the sole contribution of the 171R allele in PrPSc formation should be taken into account, though it appears that atypical/Nor98 scrapie does not transmit under field conditions and that experimental infection with BSE in 171R allele carriers is facilitated by intracerebral inoculation and disease manifests only after long incubation periods (5, 19, 28).

In conclusion, our results strongly support current genetic breeding programs in sheep aiming at eradication of classical scrapie with the concurrent potential benefit of preventing BSE infection.

ACKNOWLEDGMENTS

We are grateful to Geert-Jan Willems for skilled assistance in MAb 6C2 characterization. We are grateful to B. Jones and G. Hill from the Microbiological Services Department at the Institute for Animal Health, Compton, United Kingdom, for assistance in the generation of MAbs. Recombinant 94-233PrP was kindly provided by Andrew C. Gill (The Roslin Institute, University of Edinburgh). The help of H. Leroux and C. Rossignol in processing BSE tissues was greatly appreciated, as was the support of the INRA-PFIE level 3 animal facility. We thank DEFRA for providing “TSE-free” Pol Dorset and Suffolks NZ sheep. These investigations were largely supported by the Dutch Ministry of Economic Affairs, Agriculture and Innovation, projects WOT-01-002-001.01 and WOT-01-002-001.05. Experimental BSE and scrapie samples were generated within EU project BSE in sheep (QLRT-2001-01309), led by Olivier Andreotti, INRA-Toulouse-ENVT. Antibody characterization of FH6 was supported by the United Kingdom-Netherlands Partnership Programme in Science.

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