Neuronal accumulation of abnormal prion protein in sheep carrying a scrapie-resistant genotype (PrPARR/ARR)

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The susceptibility of sheep to scrapie infection is influenced by prion gene alleles, which are modulated by polymorphic variations corresponding to amino acid positions 136, 154 and 173 of the prion protein (PrP). As no unquestioned report of a diseased sheep carrying homozygous alleles encoding alanine, arginine and arginine (PrPARR) at these sites has been published to date, sheep of this genotype are believed to be scrapie resistant. After the introduction of large-scale rapid testing for scrapie, a number of so-called ‘atypical’ scrapie cases have been found in Germany and elsewhere. Among those cases were two supposedly scrapie-resistant sheep. Brain samples from these animals tested positive for abnormal PrP (PrPSc) in one of four rapid tests available. Moreover, scrapie-associated fibril (SAF)-immunoblotting and immunohistochemistry, which are the generally accepted diagnostic techniques for scrapie, revealed prominent PrPSc deposition in the cerebellum. SAF immunoblotting also revealed PrPSc deposition in the obex, frontal cortex and brainstem. Transmission experiments to investigate the infectivity of scrapie propagated from these sheep have been initiated.

INTRODUCTION

Scrapie in sheep is a transmissible spongiform encephalopathy (TSE), a group of infectious conditions closely associated with the deposition of abnormal prion protein (PrPSc) in the central nervous system. Incubation times in scrapie can be months or years before clinical symptoms such as neurological dysfunction and chronic and fatal wasting of the animal are observed. Clinical symptoms may depend on the infectious scrapie strain involved and on the breed and prion protein (PrP) genotype of the affected sheep. To date, there is no live animal test that is applicable during the scrapie incubation period to sheep of all PrP genotypes. Moreover, the under-reporting of clinical cases is thought to be high; therefore, the true prevalence of scrapie can only be deduced vaguely from incidence rates found by random testing of fallen stock and slaughtered animals.

The diagnostic methods currently applied to detect a TSE infection (BSE rapid tests as well as confirmatory methods) are based on the detection of PrPSc. In contrast to its cellular counterpart (PrPc), PrPSc is partially proteinase K (PK) resistant and forms scrapie-associated fibrils (SAFs) because of its high hydrophobicity (Diringer et al., 1983; Hope et al., 1986; Oesch et al., 1985; Lehmann & Harris, 1995). The four commonly used rapid tests as well as the confirmatory methods [SAF immunoblot and immunohistochemistry (IHC)] that have been recommended by the Office International des Epizooties (OIE) apply polyclonal or monoclonal antibodies to detect PK-treated PrPSc accumulated in the brains of TSE-affected animals.

In April 2002, an obligatory large-scale rapid-testing programme on slaughtered and fallen sheep and goats was implemented for monitoring purposes in the European Union (EU) and has revealed considerable numbers of scrapie cases in many member states. Because of the theoretical risk of transmission of the BSE agent to sheep and goats, the eradication of scrapie has become a high priority in the EU. These two infections cannot be distinguished by clinical symptoms or common diagnostic methods alone. Unequivocal discrimination requires comparison of the biochemical properties of the PrPSc or strain typing by lesion-profile scoring, which is performed by mouse bioassay in three conventional mouse lines (Bruce et al., 1996; Fraser & Dickinson, 1968). It is generally accepted that the susceptibility of sheep to scrapie is directly linked to particular allelic polymorphisms of PrP. Sheep carrying alleles encoding valine/arginine/glutamine (PrPVRQ) or alanine/arginine/glutamine (PrPARR) at amino acid positions 136, 154 and 171 of PrP are highly
susceptible, whereas alleles encoding alanine/arginine/arginine (PrP<sup>ARRR</sup>) seem to protect against this disease, particularly when homozygous (Goldmann et al., 1990; Hunter, 1996, 1997; Hunter et al., 1997). With one questioned exception (Ikeda et al., 1995), scrapie has never been diagnosed in a PrP<sup>ARRR</sup> homozygous sheep. In the UK, France, The Netherlands and many other EU member states, large genotyping and breeding programmes have been started in order to increase the number of so-called scrapie-resistant sheep (Arnold et al., 2002; Dawson et al., 1998). In scrapie-affected sheep flocks, recent eradication strategies rely on the removal of sheep that are considered genetically susceptible and on the selective breeding of so-called scrapie-resistant animals.

METHODS

Rapid testing. Rapid tests for BSE with kits of the following brands were done according to the manufacturers’ instructions: Platelia (Bio-Rad); Enfer TSE kit version 2.0 (Enfer Scientific); Prionics Check Western (Prionics); Prionics Check LIA (Prionics).

Confirmatory testing: SAF immunoblot. For preparation of SAFs, a 10% (w/v) homogenate was prepared of 1 g brainstem material from the obex region in 0-01 M sodium phosphate buffer, pH 7-4, containing 10% (w/v) sarcosine, 0-5 mM PMSF and 0-5 mM N-ethylmaleimide. After a preliminary centrifugation for 30 min at 20 000 × g to pellet residual debris, the supernatant was transferred into a new centrifuge tube and centrifuged for 135 min at 220 000 × g. Pellets were resuspended in 3 ml 0-015 M Tris/HCl pH 7-4 and incubated for 15 min at 37 °C and twice the sample volume of 15% (w/v) potassium iodide/high-salt buffer containing 0-1 M sodium thiosulphate pentahydrate, 0-5 mM Na<sub>2</sub>SO<sub>3</sub>. After a preliminary centrifugation for 60 min at 37 °C, supernatant was centrifuged through a 20% sucrose gradient for 30 min at 220 000 × g. Finally, sections were dehydrated automatically with pressure and vacuum at 35 °C and through a series of ethanol solutions and embedded in paraffin blocks. Sections (3 μm) were then prepared and IHC staining with the PrP-specific mAb L42, binding to an epitope at aa 145–163 of ovine PrP (Harmeyer et al., 1998), or SAF 70, binding to an epitope at aa 142–160 of hamster PrP (Demart et al., 1999), was done in an automated stainer. This procedure included a pre-treatment for 15 min in 98% formic acid followed by a 5 min incubation in tap water, 30 min in SBF and washing twice in PBS for 5 min before placing the slides into a Ventana Discovery autostainer. The automated staining protocol included a heat treatment at 95 °C for 12 min followed by a protease treatment for 12 min at 42 °C. After blocking the slides in 30% goat serum, they were incubated with primary antibody for 20 min at room temperature. This was followed by a washing procedure, incubation for 2 min with biotinylated Ig (Ventana), washing again and then incubation with streptavidin–horseradish peroxidase for 8 min. Signals were visualized with the diaminobenzidine detection system and hydrogen peroxide. Finally, sections were counterstained with haematoxylin and blueing reagent. All reagents used in this protocol were supplied by Ventana. As controls, brain sections from the obex region and cerebellum from a TSE-negative sheep and from a sheep with classical scrapie were used.

Determination of PrP allele. PrP alleles of the diseased sheep were determined by sequencing and by PCR-RFLP (Lu¨ hken et al., 2004). Briefly, genomic DNA was extracted from brain samples with a commercial kit (QiAamp DNA kit) followed by PCR amplification. To generate templates for sequencing, primers 5′-TGCGGTTGTGTTGGTGCTGCC-3′ (anti-sense; nt 22841–22861) for amplification of a 682 bp fragment and primers 5′-AAGCAAGAAGATGAGACACCACC-3′ (antisense; nt 23127–23148) for amplification of a 544 bp fragment were used in reaction mixtures comprising, in a volume of 50 μl, approximately 200 ng genomic DNA, 20 pmol of each primer, 5 mM of each dNTP, 20 mM MgCl<sub>2</sub> and 0-125 U Taq polymerase in onefold reaction buffer with the following PCR conditions: denaturation at 94 °C for 1-5 min, 40 amplification cycles of denaturation at 94 °C for 15 s, annealing at 59 °C (682 bp fragment) or 60 °C (544 bp fragment) for 20 s and extension at 72 °C for 45 s, followed by a final 5 min extension at 72 °C. The PCR fragments were used directly in sequencing reactions or restriction enzyme digestions for determination of the DNA codons at positions 136, 154 and 171 of the ovine PrP. For sequencing, each of the four PCR primers was reused, which resulted in sequences covering the complete coding region of exon 3 of ovine PRNP.

RESULTS AND DISCUSSION

In this paper we report the diagnosis of two scrapie cases in sheep carrying the supposedly scrapie-resistant PrP<sup>ARRR</sup> genotype. Both showed deposition of PrP<sup>Sc</sup> in their brains. In a more recent case, designated ARR II, a slaughtered 2-year-old sheep that had not shown any clinical symptoms was routinely tested in a regional state laboratory as part of the German scrapie surveillance programme and was found weakly reactive by the Bio-Rad Platelia test with absorbance values of 0-205 (cut-off 0-234) and 0-342/0-392 (cut-off 0-241) in the obex. The sample was subsequently submitted to the German National Reference Laboratory (NRL) for confirmatory testing. So-called SAF immunoblotting (as recommended by the OIE) (Anonymous, 2000) and the EU Commission (Anonymous, 1994) was done. The obex sample repeatedly gave a banding signal that resembled that of scrapie PrP<sup>Sc</sup>; however, at least five bands including the different PrP glycosylation forms and additional PrP
fragments were visible (Fig. 1a). A band of approximately 12 kDa was also observed that has been described for atypical scrapie cases in Norway (Fig. 1b) (Benestad et al., 2003). It was also noted that the quantities of the three PrP bands did not completely match those usually found in ‘typical’ scrapie cases with a dominant upper (diglycosylated) band, a weaker middle (monoglycosylated) band and only a faint lowest (unglycosylated) band. Instead, in the case described here, the lowest band was more prominent than the middle band, so that the signals of the diglycosylated and the unglycosylated PrP bands were of similar strengths. Moreover, the PrP deposition in this atypical scrapie case were less PK-resistant than the PrP found in classical cases (Fig. 2).

**Fig. 1.** Detection of PrP\textsuperscript{Sc} deposition in obex, frontal cortex and cerebellum (a) of the second sheep of genotype PrP\textsuperscript{ARR/ARR} (case ARR II) and the obex region of ARR cases I and II (b). Brain samples (1 g) were processed according to the SAF immunoblotting protocol with (+) or without (−) PK treatment, as indicated. Samples were then separated by SDS-PAGE and immunoblotted with mAb L42 as the detection antibody. Antibody binding was visualized with goat anti-mouse–AP and CDP-Star chemiluminescence substrate. Signals were visualized using the VersaDoc Imaging system (Bio-Rad) with Quantity One software. Positions of molecular mass markers are indicated (M). Interestingly, the PrP banding pattern does not match that of a ‘typical’ case as at least five bands of PrP and its fragments, including a 12 kDa band, are visible. This banding pattern is reminiscent of the Nor98 scrapie cases.

**Fig. 2.** Comparison of PK sensitivity of PrP\textsuperscript{Sc}. To compare the resistance of PrP\textsuperscript{Sc} derived from scrapie samples with concordant results in all tests with that of samples with incongruent results, SAF preparations were treated with PK concentrations ranging from 10 to 500 μg ml\textsuperscript{-1}. PrP\textsuperscript{C} from an uninfected sheep control was completely digested by 10 μg ml\textsuperscript{-1}; PrP\textsuperscript{Sc} from a sample with concordant results (S3/02) still gave a strong signal after incubation with 500 μg ml\textsuperscript{-1} for 60 min at 37 °C; PrP\textsuperscript{Sc} from a sample with incongruent results (S14/03) was almost completely digested after incubation with 250 μg ml\textsuperscript{-1}. MAP L42 was used as the detection antibody.
A sample from the obex region was also taken and subjected to IHC staining with mAbs L42 and SAF 70 but no PrP-specific signals were visible. Incidentally, several other brain regions were available from this animal and were analysed by rapid testing, SAF immunoblotting and IHC. For the obex region, a different sample had to be taken for retesting with the Bio-Rad Platelia test at the NRL, and gave an absorbance value of 0.767 (Table 1). These studies revealed PrPSc depositions, with the largest amounts in the cerebellum, and smaller or borderline amounts in the frontal cortex, diencephalon and obex regions (Table 1, Fig. 3). Most strikingly, IHC with mAbs L42 and SAF 70 on the cerebellum of this case revealed a very strong signal for PrPSc deposition in the molecular cell layer, whereas no staining was found in other regions of the central nervous system (Fig. 3). This is in contrast to what has been described for classical scrapie cases, where PrPSc deposition is detectable mainly in association with cellular membranes of neurons and astrocytes in the medulla oblongata and pons but no staining is seen in the cerebellum (Van Keulen et al., 1995), which is in accordance with what has recently been described for atypical scrapie cases in Norway designated Nor98 (Benestad et al., 2003). Surprisingly, this case was not detected by the Prionics Check Western test or the LIA rapid test, an observation that has also been reported for a number of ‘atypical’ scrapie cases (Buschmann et al., 2004) recently found in Germany, France, Norway (Benestad et al., 2003) and elsewhere. The reason for this non-homogeneous detection has been analysed for a number of German and French scrapie cases (Buschmann et al., 2004) and it seems to be more dependent on the sheep isolate than on the antibodies applied for PrP detection. To get a second opinion on our interpretation, a formalin-fixed obex sample and the SAF immunoblotting and IHC testing results were submitted to the Community Reference Laboratory (CRL) at the Veterinary Laboratories Agencies, Weybridge, UK. The CRL confirmed the absence of IHC staining of the obex, but from the positive SAF immunoblotting and positive IHC results in other brain areas concluded that this would be sufficient to support the confirmation of this case as TSE positive.

As the head of this animal was available for further sampling, the lymphoid tissue adherent to the nictitating membrane as well as the mandibular and retropharyngeal lymph nodes were examined by IHC with mAbs L42 and SAF 70. However, no PrP-specific staining was detectable in these tissues (data not shown). This is in accordance with what has been described previously for scrapie-affected sheep carrying only one ARR allele (Van Keulen et al., 1996; Andréoletti et al., 2000).

Following this diagnosis, all animals in the flock from which the affected sheep were derived were genotyped, and animals carrying no PrPARR allele or the PrPVRQ allele in the homozygous or the heterozygous form were culled. No other scrapie case was found by rapid testing (Bio-Rad Platelia) within this selected group. This may result at least partly from the fact that no PrPSc was detectable in the analysed lymphoid tissue of this animal, which reduces the risk of horizontal scrapie transmission within a flock.

In the course of retrospective genotyping of all German scrapie cases, DNA was prepared from brain tissue (three independent samples) and from masseter muscle of the scrapie-diagnosed animal and sequenced by RFLP analysis. Surprisingly, all tests consistently gave the PrP genotype of this animal to be PrPARR/ARR. Genotyping of this sheep was repeated by another laboratory and the PrPARR/ARR allele was again confirmed. Other than the amino acid substitution at codon 171 (QQ/RR), no further nucleotide variations were observed comparing the sequence obtained, covering the complete coding region of exon 3 of PRNP, with GenBank sequence U67922. This is the second time that a PrPARR/ARR genotype has been found in a scrapie case from Germany. A previous case, designated ARR I, which was also discovered retrospectively by genotyping, occurred in an 8–10-year-old female sheep that was sent for rendering, and was tested using the Bio-Rad Platelia rapid test as a routine sample. The only available brain-tissue

Table 1. Detection of PrPSc in different brain regions of the ARR II case using rapid tests and confirmatory methods at the German TSE reference laboratory

<table>
<thead>
<tr>
<th>Brain region</th>
<th>Bio-Rad Platelia (cut-off 0.294)</th>
<th>Prionics Check Western</th>
<th>Prionics Check LIA</th>
<th>SAF immunoblot</th>
<th>IHC</th>
</tr>
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<tbody>
<tr>
<td>Obex</td>
<td>0.767</td>
<td>–</td>
<td>76</td>
<td>++</td>
<td>–</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>1.424</td>
<td>–</td>
<td>74</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Frontal cortex</td>
<td>1.206</td>
<td>–</td>
<td>91</td>
<td>+</td>
<td>+/-</td>
</tr>
</tbody>
</table>

Note that a fresh obex sample was taken for the Bio-Rad test at the NRL as no residual homogenate was available from the initial testing at the regional laboratory. Results are absorbances for the Bio-Rad Platelia test (cut-off 0.294), PrPSc signal for the Prionics Check Western and relative light units for the Prionics Check LIA (cut-off 2791). Scores are: –, no signal; +/-, very weak signal; +, weak signal; ++, strong signal; ++++, very strong signal.
Fig. 3. IHC staining for PrP<sup>Sc</sup> deposition in the obex (c), cerebellum (e–h), thalamus (i) and frontal cortex (j) of case ARR II. Obex (a) and cerebellum (d) sections from a normal animal served as negative control, whereas an obex section of a classical scrapie case (b) was used as positive control. mAb L42 was used throughout the experiment except for (f), which was stained with mAb SAF 70 in order to emphasize the specificity of the staining. Note the heavy IHC staining, as indicated by precipitation of brown substrate (diaminobenzidine), indicating PrP<sup>Sc</sup> deposition in the molecular layer (e–h) also extending into the granular layer (g, h) of the cerebellum. Bars, 50 μm.
sample from this case was heavily autolysed and of a paste-like consistency so that the obex region could scarcely be determined. The Platelia readings on the assumed obex sample were borderline reactive (absorbance in duplicate readings 0·231/0·554) in the first set of experiments and low reactive in the second set of experiments (absorbance in duplicate readings 0·426/0·492). However, no positive result was obtained when the Prionics Check Western or LIA or the Enfer rapid test were used. Accumulation of PrPSc was also found by SAF immunoblotting in two independent experiments (Fig. 1b). Again, the banding patterns did not match those of ‘typical’ scrapie. IHC staining with mAbs L42 or SAF 70 on the assumed obex was negative, a result that could also have been because of the heavy autolysis or a sampling artefact. The suspicion of scrapie was eventually confirmed on the basis of the Bio-Rad Platelia and the SAF immunoblotting results. All sheep in the herd of origin were culled irrespective of their genotype, but no other sheep was found to be positive.

In this case, DNA purified from the reactive brain was sequenced and PrPARR/ARR allele was determined. To confirm this, another DNA preparation was genotyped, again in the same laboratory and in parallel in an independent laboratory. All investigations confirmed the first genotyping results of ARR homozygosity. Again, no nucleotide variations other than the amino acid substitution at codon 171 (QQ/RR) could be observed by comparing the sequence obtained with GenBank sequence U67922.

For both of the potential ARR scrapie cases, mouse bioassays have now been initiated to investigate the presence of inherent TSE infectivity. RIII, C57Bl, VM95 and ovine PrPc (PrPARR) -overexpressing transgenic mice have been inoculated intracerebrally with cerebellar and/or obex homogenates (10 %, w/v). Results from these studies are not expected to be available before the middle of 2005.

It is well established that PrP ARR allele homozygosity does not confer absolute resistance to TSE experimental challenge. PrPARR/ARR sheep experimentally exposed to BSE by intracerebral inoculation develop clinical signs of a TSE infection (Houston et al., 2003). PrPSc found in these animals displayed the same glycoform pattern as found in diseased sheep of the susceptible PrP ARQ homozygous genotype. Moreover, cell-free conversion of PrP ARR using ‘typical’ scrapie as seed is possible, albeit at a much lower efficiency (Bossers et al., 2000).

There has been no previous unquestioned report of a natural scrapie case in a PrPARR/ARR sheep, although a large number of diseased animals have been genotyped. However, the large majority of scrapie cases have been confirmed previously by IHC staining of PrPSc in the obex, which is considered the most reliable diagnostic marker of scrapie (Anonymous, 2004). Only in a few instances was SAF immunoblotting carried out, because this requires a larger quantity of sample and is more time-consuming. After the introduction of the scrapie-monitoring programme in the EU, ‘in which obex samples are now being tested using rapid tests, ‘atypical’ scrapie cases are being detected more frequently. It is intriguing to see that ‘atypical’ scrapie cases have been uncovered recently in which PrPSc deposition at the level of the obex is faint or absent. As only the obex or brainstem is sampled for this programme, reassessing these cases by analysis of other brain areas is often impossible. Therefore, ‘atypical’ scrapie cases may have been previously under-reported. However, it must be noted that the infectious nature of this novel scrapie type still has to be confirmed by transmission experiments. A similar situation may exist for yet unrecognized scrapie cases in PrP ARR/ARR animals, which may display a distinct PrPSc deposition topology in the brain and a distinct biochemical glycotyping pattern. This novel phenotype may originate from a particular PrP genotype, from a peculiar scrapie strain or from the combination of the two.

It is interesting to see that SAF immunoblotting of the obex or cortex gave a positive result, like the Bio-Rad Platelia, while IHC staining for PrPSc was negative. It could be argued that IHC is much less sensitive at recognizing these particular cases. Moreover, our own results indicate that SAF preparation is in general more sensitive to dilution of positive samples than IHC staining (A. Buschmann and others, unpublished results). However, the heavy PrP staining in the cerebellum proves that this method is in principle equally suitable for detection of these cases.

Taken together, the findings reported here indicate that sheep homozygous for the PrPARR allele may not be fully resistant to natural scrapie infections and may exhibit diagnostic features that fit the most recently discovered ‘atypical’ scrapie case definition. ‘Atypical’ scrapie cases occur predominantly in sheep carrying a scrapie-resistance PrP allele in heterozygous or homozygous form. If transmission studies indeed show that the PrPSc depositions in these cases are infectious and that such infections are able to spread from sheep under natural conditions, these findings would question the large-scale sheep genotyping and scrapie-resistant breeding programmes that have been introduced in several EU member states over the last 5 years.

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