Immunohistochemical characteristics of disease-associated PrP are not altered by host genotype or route of inoculation following infection of sheep with bovine spongiform encephalopathy

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It has previously been reported that disease-associated prion protein (PrP\textsuperscript{d}) derived from natural scrapie and from sheep infected experimentally with bovine spongiform encephalopathy (BSE) differed in respect of their immunohistochemical and immunoblotting properties. For BSE, however, these initial observations were restricted to orally challenged sheep of the ARQ/ARQ PrP genotype. Here, extended examinations were performed on 28 sheep that developed neurological signs after BSE experimental infection by one of three routes. Intracerebrally infected ARQ/ARQ sheep showed more widespread and abundant accumulations of PrP\textsuperscript{d} in tissues of the lymphoreticular system (LRS) than VRQ/VRQ animals, whereas no peripheral PrP\textsuperscript{d} was detected in ARR/ARR sheep. The intensity and dissemination of PrP\textsuperscript{d} accumulation in LRS tissues were less than those found previously in orally dosed sheep. AHQ/AHQ sheep challenged orally and ARQ/AHQ and ARQ/ARQ animals infected intravenously showed similar LRS-tissue PrP\textsuperscript{d} distributions and levels to those of ARQ/ARQ sheep infected intracerebrally. The patterns of intra- and extracellular immunoreactivity to different PrP antibodies in brain and LRS tissues and the immunoblotting characteristics of PrP\textsuperscript{res} from brain samples remained constant, irrespective of the route of inoculation and the PrP genotype, and were the same as described previously for ARQ/ARQ sheep dosed orally with BSE. These results suggest that the intracellular truncation of BSE PrP\textsuperscript{d} and the proteinase K cleavage site of BSE PrP\textsuperscript{res} are not altered by PrP genotype or by route of inoculation and that, therefore, screening tests based on these properties can be applied to identify potential sheep BSE cases occurring naturally.

INTRODUCTION

The transmissible spongiform encephalopathies (TSEs) are a group of progressive neurological disorders that affect a number of animal species and humans and include scrapie of sheep and goats and bovine spongiform encephalopathy (BSE). A variant form of Creutzfeldt–Jakob disease (vCJD) of humans was initially described in 1996 and is thought to have been the result of BSE-infected cattle tissues entering the human food chain. The nature of the agent causing TSEs is still in dispute, but an abnormal isoform of a host-encoded cell-surface glycoprotein (prion protein, PrP\textsuperscript{*}) accumulates during disease and is associated closely with infectivity. This disease-associated form of PrP is insoluble and partially protease-resistant (PrP\textsuperscript{res}). Disease-associated PrP may also be revealed by immunohistochemical (IHC) methods that do not involve enzymic pre-treatment, therefore requiring the use of different terminology (PrP\textsuperscript{d}).

Since the ban on the use of ruminant feed in the 1990s, the prevalence of BSE in cattle in the UK has declined steadily and it has recently been suggested that the incidence of vCJD cases may have peaked in 2000 (Andrews et al., 2003). However, sheep may also have been exposed to BSE-contaminated meat and bone meal during the mid-1980s and mid-1990s (Butler, 1998) and previous studies have shown that BSE may be transmitted readily to sheep by oral, intravenous and intracerebral routes (Foster et al., 1993; Jeffrey et al., 2001c; Houston et al., 2003). The possibility that consumption of BSE-infected sheep meat may pose a risk to humans has prompted urgent research into this area. As sheep originally exposed to cattle BSE
would not be in production at present and as the feeding of meat and bone meal to ruminants is no longer legal, any ovine BSE infection could only be maintained by transmission between sheep. Whilst maternal and horizontal (except from contaminated feedstuffs) methods of transmission of cattle BSE do not occur or are epidemiologically insignificant (Wilesmith et al., 1997), scrapie is maintained endemically in flocks through sheep-to-sheep contagion. However, as the distribution of PrP\textsuperscript{d} in tissues of sheep infected orally with BSE (Jeffrey et al., 2001c) resembles that of natural sheep scrapie more closely than that of cattle BSE (Van Keulen et al., 1996, 2000; Jeffrey et al., 2001b; Heggebo et al., 2002), the epidemiological features of BSE in cattle cannot be taken as an example for any potential naturally occurring sheep BSE.

Detectable infectivity in cattle with BSE is mainly confined to tissues that may be excised from the carcass, thus rendering the risk to man via the food chain low (Wells et al., 1998). Evidence obtained from mouse bioassay has shown that, in experimental cattle BSE, infectivity is present in the distal ileum between 6 and 18 months post-infection, in the central nervous system (CNS) and dorsal root ganglia at 32 months post-infection and in the peripheral nervous system between 32 and 40 months post-infection (Wells et al., 1998), with the earliest onset of clinical signs taking place at 35 months post-infection. This pattern is in marked contrast to BSE infection of sheep. By using IHC detection of PrP\textsuperscript{d} as a marker (Prusiner, 1999), infectivity has been found in the intestinal Peyer’s patches as early as 5 months post-infection (Jeffrey et al., 2001c). Subsequently, spread to the enteric nervous system and spinal cord occurs after 10 months and widespread dissemination throughout the lymphoreticular and peripheral nervous systems by 21 months post-infection (Jeffrey et al., 2001c). This widespread infectivity of tissues in experimentally BSE-infected sheep (and also in natural scrapie) would make successful excision of high-risk tissues from the carcass impractical.

In previous studies, we have shown that oral BSE in sheep of the ARQ/ARQ genotype can be distinguished from natural sheep scrapie by differences in the immunolabelling patterns of phagocytic cells and neurons with a panel of PrP antibodies (Jeffrey et al., 2001a, 2003; González et al., 2003). The intracellular aggregates of PrP\textsuperscript{d} in tingible body macrophages (TBMs) and in glial cells are located in lysosomes (Jeffrey et al., 1994, 2000), where they are acted upon by endogenous proteases, so that the full-length protein will be truncated and partially digested. The site of truncation within the N terminus of PrP appears to be different for sheep scrapie and experimental sheep BSE, giving rise to distinct IHC patterns. These data have recently been supported by Western blot studies on brain (Stack et al., 2002; Lezmi et al., 2004; Thuring et al., 2004), which suggest that, after digestion by exogenous enzymes, the BSE PrP\textsuperscript{res} molecule is shorter than that obtained from all sheep scrapie sources so far tested. Preliminary studies of the brains of three BSE-affected ARR/ARR sheep also showed similar features (Houston et al., 2003).

In order to determine whether the truncation patterns of PrP\textsuperscript{d} and/or PrP\textsuperscript{res} reported previously in ARQ/ARQ sheep challenged orally with BSE are influenced by route of infection or by the genotype of the host, we have examined six groups of sheep with different PrP genotypes that had been challenged orally, intracerebrally (IC) or intravenously (IV) with BSE agent. In the present study, we show that the route of infection and the host PrP genotype do not affect the immunolabelling and Western blotting properties of sheep BSE PrP\textsuperscript{d}.

**METHODS**

**Animals and experimental details.** Fifteen sheep of three different genotypes (ARQ/ARQ, VRQ/VRQ and ARR/ARR, n = 5 in each) were infected experimentally by the IC route as described by Foster et al. (1993). Briefly, 0.5 ml of a 10% brainstem homogenate made from a pool of three confirmed cattle BSE cases was delivered at a point approximately 4 cm caudal to the frontal crest and 1 cm lateral to the midline at a depth of 2–3 cm via a 5 cm, 21 gauge needle. Thirteen of the IC-infected animals were of the Cheviot breed and two were Suffolk sheep (Table 1). IV BSE infection was carried out in ten Cheviot sheep. Five of them (ARQ/AHQ PrP genotype) were challenged with 20 ml of a 1% dilution of the same cattle-brain pool as was used for IC challenge. The other five (ARQ/ARQ) were inoculated with 400–450 ml whole blood (or its buffy

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Breed</th>
<th>Route</th>
<th>Incubation period (mean ± SEM) (days)</th>
<th>No. animals</th>
<th>No. tissues positive</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Tonsil</td>
</tr>
<tr>
<td>ARQ/ARQ</td>
<td>Cheviot</td>
<td>IC</td>
<td>608 ± 17</td>
<td>5</td>
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</tr>
<tr>
<td>VRQ/VRQ</td>
<td>Cheviot</td>
<td>IC</td>
<td>1068 ± 9</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>ARR/ARR</td>
<td>Cheviot</td>
<td>IC</td>
<td>1274 ± 124</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>ARR/ARR</td>
<td>Suffolk</td>
<td>IC</td>
<td>1274 ± 124</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>ARQ/ARQ</td>
<td>Cheviot</td>
<td>IV</td>
<td>565 ± 16</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>ARQ/AHQ</td>
<td>Cheviot</td>
<td>IV</td>
<td>716 ± 22</td>
<td>5</td>
<td>5</td>
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<tr>
<td>AHQ/AHQ</td>
<td>Cheviot</td>
<td>Oral</td>
<td>626 ± 21</td>
<td>3</td>
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</tr>
</tbody>
</table>
coat equivalent diluted in 50 ml PBS) obtained from three donor Cheviot sheep that had previously been exposed to BSE by the oral route (see below). The IV experiments have been described in detail by Hunter et al. (2002). Three AHQ/AHQ Cheviot sheep were challenged by the oral route with a 10% homogenate of a cattle-brain BSE pool, as described previously (Jeffrey et al., 2001c).

Sheep genotypes were assigned following sequencing of the PrP gene coding region, as described by Baylis et al. (2000), and are represented by single-letter codes at codons 136, 154 and 171, respectively. All of the 28 animals used in this study had developed clinical signs of TSE, with group mean incubation periods ranging from 565 to 1274 days (Table 1). For comparison with BSE, tissues obtained from naturally affected ARQ/ARQ Suffolk sheep from a heavily scrapie-infected flock (Jeffrey et al., 2001b) were subjected to the same laboratory examinations as experimental BSE cases.

**Immunohistochemistry.** After developing clinical signs of TSE, animals were euthanized and post-mortem examination was performed. Samples of medulla oblongata at the level of the obex, spleen, palatine tonsil and mesenteric lymph node (MsLN) were fixed in neutral 10% phosphate-buffered formalin, trimmed, post-fixed and embedded according to standard procedures. Sections (5 μm thick) were cut on a microtome, mounted on treated glass slides (Superfrost Plus; Menzel-Glaser) and dried overnight at 37 °C.

IHC methods have been described previously (González et al., 2002, 2005). Briefly, tissue sections were immersed in 98% formic acid for 15 min, followed by 30 min autoclaving at 121 °C in 0.2% citrate buffer, pH 6.4. Incubation with the primary antibody was performed overnight at 4 °C and the rest of the IHC protocol was performed by a commercial immunoperoxidase technique (VECTASTAIN Elite ABC; Vector Laboratories), tissue sections being finally counterstained with Mayer’s haematoxylin. Serial sections of each tissue sample were immunolabelled with five different PrP antibodies, namely R145 (rat mAb at 1/4000 dilution), P4 (mouse mAb at 1/20000 dilution), 521 and 505 (rabbit polyclonal antisera at 1/10 000 and 1/6000 dilutions, respectively) and BG4 (mouse mAb at 1/2000 dilution). The PrP epitope affinity of these antibodies has been referred to previously (Jeffrey et al., 2001a, 2003; Gonzaález et al., 2003). BG4 was raised against recombinant bovine PrP and mapped to a sequence between amino acids 45 and 57 by Pepsan analysis (Geysen et al., 1984). The epitope recognized by the P4 antibody (amino acids 93–99 of ovine PrP) has recently been determined precisely (Thuring et al., 2004).

Control tissues used in each IHC run were as follows. For brain, positive controls included samples of oesophagus from a clinically sick sheep previously challenged with ovine BSE and from a Suffolk sheep naturally affected with scrapie. For lymphoid tissues, positive controls consisted of samples of tonsil or MsLN from an ARQ/ARQ sheep challenged orally with BSE and from an ARQ/ARQ Suffolk sheep with natural scrapie. As negative controls, samples of oesophagus and LRS tissues from a normal, unaffected ARQ/ARQ sheep were used.

**Morphometric methods and statistical analysis.** Morphometric estimations of the amount of PrP<sub>d</sub> accumulation were performed for tonsil, MsLN and spleen on all sheep included in the study. To determine the distribution and intensity of PrP<sub>d</sub> accumulation in these tissues, two parameters were scored: the percentage of positive follicles and the magnitude and distribution of PrP<sub>d</sub> labelling within follicles. To avoid biases arising from variation in follicle size or in the site of PrP<sub>d</sub> accumulation, only follicles in which distinct light and dark zones could be seen were counted. The magnitude of labelling was scored as follows: 0, no labelling; 1, labelling confined to only one or two TBMls in the light zone; 2, TBM labelling throughout the light zone; 3, TBM labelling in both the light and dark zones; 4, TBM labelling in both the light and dark zones and weak labelling associated with follicular dendritic cells (FDCs) present in the light zone; 5, as for 4, but intense FDC-type labelling in the light zone. These labelling patterns are interpreted to indicate a progressive increase in the magnitude and/or duration of follicular infection. For each LRS tissue examined, a mean value was obtained from those follicles that actually showed PrP<sub>d</sub> immunolabelling.

Comparisons of morphometric data, both percentage of positive follicles and magnitude of immunolabelling, obtained from the different sheep groups examined were performed by Student’s two-sample test using a commercially available statistical package (InStat; Graphpad Software).

**Western blotting.** Western blotting was performed on samples of brain from all IC-challenged sheep in order to detect PrP<sub>res</sub>. PrP<sub>res</sub> was extracted by using modifications of the methods described by Hope et al. (1986, 1988) as follows: 0.2-g brain tissue was homogenized in 2.0 ml 0·01 M sodium phosphate, pH 7·4, 10% (w/v) N-lauryl sarcosinate containing 1·0 mM phenylmethylsulphonyl fluoride and 1 mM N-ethyl maleimide. The suspension was centrifuged at 13 000 g for 20 min. After discarding the pellet, the supernatant was centrifuged at 215 000 g for 150 min at 10 °C. The supernatant was discarded and the pellet was resuspended in 0·6 ml deionized water and shaken at 37 °C for 30 min. The sample was then adjusted to a volume of 1·8 ml with deionized water and to an ionic concentration of 0·6 M potassium iodide, 6 mM sodium thiocyanate, 1% (w/v) N-lauryl sarcosinate and 10 mM sodium phosphate, pH 8·5. The sample was then treated with 50 μg proteinase K (PK) ml<sup>−1</sup> and incubated with agitation at 37 °C for 60 min. The PK-treated fraction was sedimented through a 2 ml cushion of 20% sucrose, 0·6 M potassium iodide, 6 mM sodium thiocyanate, 1% (w/v) N-lauryl sarcosinate and 10 mM sodium phosphate, pH 8·5, by centrifugation at 285 000 g for 90 min at 10 °C. The pellets were dissolved in 23·4 M formic acid and dried under vacuum. Peptide-N-glycosidase F (PNGase)-treated PrP<sub>res</sub> was prepared by using the method described by Collinge et al. (1996).

Samples were run on 14% Tris/glycine gels (Novex) and immunoblotted onto PVDF membranes. For detection of PrP<sub>res</sub>, two mAbs were used: 6H4 (Prionics, 2 mg ml<sup>−1</sup>) diluted 1:5000 and P4 (R-Biopharm, 1 mg ml<sup>−1</sup>) diluted 1:2500. Visualization of the blots was performed with a chemiluminescent substrate and Lumi-Film (both from Roche).

**RESULTS**

**PrP<sub>d</sub> distribution and magnitude of accumulation in LRS tissues**

It is well-known that, in some TSEs, such as sporadic CJD or BSE, and even in some scrapie-affected sheep, accumulation of PrP<sub>d</sub> in tissues outside the CNS does not occur or does so only in limited amounts. In contrast, in many other TSEs, including most natural and experimental sheep TSEs, PrP<sub>d</sub> accumulation is not confined to the brain but is widespread, especially throughout the LRS. The precise reasons for these differences are not understood. We have evaluated the amount and distribution of PrP<sub>d</sub> in a selected range of LRS tissues from sheep of different PrP genotypes challenged experimentally with BSE by different routes, in order to determine whether the route of challenge or genotype affects the involvement of the LRS.

Following IC infection, the proportion of LRS tissues.
accumulating PrP\textsuperscript{d} varied according to PrP genotype, with ARQ/ARQ sheep showing the most widespread distribution of PrP\textsuperscript{d}, ARR/ARR animals lacking detectable PrP\textsuperscript{d} in LRS tissues and VRQ/VRQ sheep showing an intermediate situation (Table 1). Statistically significantly more positive follicles were found in ARQ/ARQ sheep than in VRQ/VRQ animals in tonsil, MsLN and spleen (Table 2; Fig. 1a). In sheep of these two genotypes, the percentage of PrP\textsuperscript{d}-positive follicles was lower in the MsLN than in the tonsil ($P<0.01$), whereas the spleen did not show differences from either of the other two LRS tissues in this respect (Table 2; Fig. 1a).

When values for the magnitude of PrP\textsuperscript{d} accumulation within positive follicles were compared, a similar genotype-related effect was found. ARQ/ARQ sheep showed a greater, although not statistically significant, magnitude of follicular immunolabelling than VRQ/VRQ animals (Table 2; Fig. 1b). Accumulation of PrP\textsuperscript{d} was found to be consistently less intense in the MsLN than in the spleen or tonsil (Fig. 1b). Statistical analysis indicated significant differences in the intensity of follicular labelling between the MsLN and the spleen for ARQ/ARQ and VRQ/VRQ sheep, significant differences between the MsLN and the tonsil for both genotypes and no differences between tonsil and spleen for either of these genotypes.

Following IV or oral inoculation, sheep of all three PrP genotypes examined showed a consistent involvement of the palatine tonsil but, in agreement with data from IC-challenged sheep, the MsLN and spleen were only affected consistently in ARQ/ARQ sheep (Table 1). Both groups of IV-challenged sheep had a greater intrafollicular intensity of immunolabelling in the spleen when compared with the other two LRS tissues examined and a greater percentage of follicles accumulating PrP\textsuperscript{d} in spleen than in tonsil and/or MsLN (Table 2). Conversely, orally challenged sheep had a lower percentage of follicles accumulating PrP\textsuperscript{d} and a lower intrafollicular intensity of immunolabelling in the spleen when compared with the other two LRS tissues (Table 2). Similarly, IV-challenged sheep had a greater percentage of follicles accumulating PrP\textsuperscript{d} and intrafollicular intensity of immunolabelling in the spleen when compared with orally challenged sheep (Table 2).

### Table 2. Mean percentage of positive follicles and average modal magnitude of PrP\textsuperscript{d} accumulation in follicles of three LRS tissues according to sheep genotype and route of inoculation

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Route</th>
<th>Mean percentage of positive follicles ($\pm$ SEM)</th>
<th>Average modal magnitude ($\pm$ SEM)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Tonsil</td>
<td>MsLN</td>
</tr>
<tr>
<td>ARQ/ARQ</td>
<td>IC</td>
<td>85 ± 6.9</td>
<td>46 ± 10.2</td>
</tr>
<tr>
<td>VRQ/VRQ</td>
<td>IC</td>
<td>54 ± 12.6</td>
<td>15 ± 11.3</td>
</tr>
<tr>
<td>ARR/ARR</td>
<td>IC</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ARQ/ARQ</td>
<td>IV</td>
<td>59 ± 14.3</td>
<td>66 ± 16.1</td>
</tr>
<tr>
<td>ARQ/AHQ</td>
<td>IV</td>
<td>81 ± 13.4</td>
<td>57 ± 11.5</td>
</tr>
<tr>
<td>AHQ/AHQ</td>
<td>Oral</td>
<td>100</td>
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</table>

### PrP\textsuperscript{d} immunolabelling with different antibodies (peptide mapping) in LRS and CNS tissues

In previous studies, we have shown that, unlike scrapie, the intracellular truncation site of ovine BSE PrP\textsuperscript{d} is influenced...
by the tissue and cell types in which it accumulates, giving rise to distinct patterns of intracellular immunolabelling when different PrP antibodies are employed. By using this IHC strategy, also called epitope mapping, the inferred shortest fragment of intracellular PrPd is detected in TBM.

In contrast, in natural sheep scrapie, intracellular PrPd appears to be of the same length, regardless of the cell type in which it accumulates, and is always longer than any intracellular PrPd found in sheep BSE. In the present study, we aimed to determine whether the host PrP genotype or the route of infection could affect these IHC differential properties of ovine BSE and whether or not differentiation between ovine BSE and sheep scrapie would still be possible independently of such variables.

A summary of the intracellular labelling patterns for LRS tissues is shown in Table 3 and examples are shown in Fig. 2. As described above (see Tables 1 and 2), PrPd did not accumulate in the LRS tissues of all sheep studied, but when it did, intense, multifocal, granular accumulations could be identified throughout the light and dark zones of secondary follicles. This pattern of labelling is associated with intracellular accumulation of PrPd within TBM. When tissues were immunolabelled with the C-terminal PrP antibody R145, the TBM-associated PrPd pattern could be seen in all positive follicles and LRS tissues. However, when serial sections of these tissues and secondary follicles were incubated with PrP N-terminal antibodies (BG4, P4, 521 and 505), there was little or no discernible labelling of TBM. This antibody-dependent differential immunolabelling was not affected by the route of challenge or the host PrP genotype. In contrast to BSE-challenged sheep, LRS tissues from Suffolk scrapie-affected sheep showed labelling of TBM with all the antibodies used except BG4 (Table 3).

A summary of the intracellular immunolabelling patterns in brain is shown in Table 3. All BSE-affected sheep, regardless of their PrP genotype and route of infection, showed evidence of intracellular labelling with some antibodies, but not with others. Intraglial labelling was observed consistently with the R145 antibody as intense, granular deposits of PrPd in close proximity to the nuclei of glial cells. With the 505 antibody, a pattern of intraglial labelling similar to that of the R145 antibody was found, albeit with a lower intensity and less uniform distribution. With the 521 antibody, large granular foci adjacent to the nuclei of glial cells were not seen. However, in some sheep where intense neuropil PrPd accumulation was present, fine puncta were occasionally seen in close proximity to glial nuclei. With the P4 antibody, no granular or fine punctuate labelling was seen near glial-cell nuclei. Although there was some variation in overall magnitude, the described pattern of immunolabelling with the four antibodies tested for intraglial PrPd was the same in all BSE-infected sheep (Table 3). In contrast, Suffolk sheep with natural scrapie showed similar magnitudes of intraglial PrPd accumulation with all four antibodies tested. As in the LRS, the BG4 antibody did not label any intracellular accumulations of PrPd.

In all BSE-affected sheep, intraneuronal accumulation of PrPd was not observed with the P4 or BG4 antibodies, but was found with all three other antibodies tested (Table 3; Fig. 3). In contrast, Suffolk sheep with natural scrapie showed similar magnitudes of intraneuronal PrPd accumulation with antibodies R145, 505, 521 and P4. As described previously, the extreme N-terminal antibody BG4 did not label intracellular PrP accumulations in glia or in neurons.

**IHC of negative-control tissues**

With the PrP antibodies R145, 505, 521 and P4, no immunolabelling was detected in normal brain or LRS tissue samples. P4 provided weak labelling of light zones of secondary follicles in LRS tissues and diffuse labelling of grey matter in brain. Occasional neurons throughout the brain showed strong cytoplasmic labelling. These patterns were considered to represent PrPC (data not shown).

**Western blotting profiles of PrP**

The IHC epitope-mapping results described above suggest intracellular truncation of PrPd in phagocytic and non-phagocytic cells occurring at different sites of the N terminus.
of the abnormal protein when sheep BSE and natural scrapie are compared. Ultrastructural studies indicate that truncation takes place in lysosomes, where PrP<sup>PSE</sup> accumulates (Jeffrey et al., 1994, 2000), and is therefore likely to result from enzymic activity. Western blotting and most other rapid testing methods for diagnosis of TSEs in ruminants use PK to reveal the presence of PrP<sup>res</sup> in tissue samples. Preliminary results from Western blotting analysis of experimental sheep BSE and natural scrapie indicate that the site of truncation by this exogenous enzyme differs between

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**Fig. 2.** The PrP<sup>D</sup>-labelling pattern in secondary follicles is shown in the tonsils of two genotypes of BSE-infected sheep and one sheep scrapie-infected MsLN following labelling with the R145 (d, h, l), 505 (c, g, k), 521 (b, f, j) or P4 (a, e, i) antibodies. Serial sections through the same secondary follicle of (a, b, c, d) natural scrapie-affected ARQ/ARQ Suffolk sheep, (e, f, g, h) BSE-infected ARQ/ARQ sheep and (i, j, k, l) BSE-infected VRQ/VRQ sheep. Marked TBM labelling is also evident in the dark and light zones of all secondary follicles labelled with the C-terminal R145 antibody (d, h, l), but only the sheep scrapie node shows labelling of TBMs when labelled with antibodies to the N-terminal flexible tail of the PrP molecule: P4 (a), 521 (b) or 505 (c). No TBM labelling is evident in the dark zones of VRQ/VRQ or ARQ/ARQ BSE-infected sheep when labelled with these same N-terminal PrP antibodies (e, f, g, i, j, k). The dark zones of follicles are indicated by 'D' and by a dotted line in (f). Bars, 50 µm.
these two infections, allowing its differential diagnosis (Stack et al., 2002). In the present study, we have addressed whether or not these differential immunochemical properties of sheep scrapie and ovine BSE PrPres depend on the host PrP genotype.

Samples of BSE-affected sheep brains analysed by Western blotting using the mAb 6H4 gave strong PrPres bands in all experimental BSE sheep (Fig. 4a). A range of intensities of labelling with 6H4 and some variation in the speed of migration of PrPres was observed, but these were probably related to protein-loading levels and not to differences in PrP genotype. The apparently lower molecular mass of the unglycosylated fragment of PrPres from sheep BSE samples compared with scrapie samples (Stack et al., 2002) was observed consistently (Fig. 4a) and could be demonstrated more clearly by removing the carbohydrate side chains following treatment of the samples with PNGase (Fig. 4b). The main differential Western blot feature between sheep BSE and natural scrapie is the decreased signal found in blots labelled with mAb P4 (Stack et al., 2002). Samples of BSE-affected sheep brains analysed by Western blotting using the mAb P4 showed no significant differences between sheep of different PrP genotypes, which could be distinguished readily from scrapie controls (Fig. 4c), as they showed a much lower labelling intensity. As samples from

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**Fig. 3.** The intraneuronal labelling pattern in the dorsal (parasympathetic) nucleus of the vagus nerve is shown for three genotypes of BSE-infected sheep when labelled with the R145 (a, b, c) or P4 (d, e, f) antibodies. The ARQ/ARQ genotype is shown in (a) and (d), VRQ/VRQ genotype in (b) and (e) and ARR/ARR in (c) and (f). Marked intraneuronal labelling was conspicuous when tissues were labelled with the C-terminal antibody R145 (a, b, c). No significant intraneuronal labelling was present when tissues were labelled with the N-terminal antibody P4 (d, e, f). Bars, 50 μm.
DISCUSSION

The IHC and Western blotting findings in the present study of experimental BSE infection in sheep of several PrP genotypes challenged by three routes of inoculation are similar to results obtained in earlier studies, where BSE infection was confined to orally challenged ARQ/ARQ sheep (Jeffrey et al., 2001a, 2003; Stack et al., 2002; Thuring et al., 2004). We have shown that the ‘in vivo’ and ‘in vitro’ truncation site of sheep BSE PrP<sup>D</sup> and PrP<sup>RES</sup>, as revealed by IHC and immunoblotting, respectively, is independent of PrP genotype, sheep breed or route of challenge and differs from that of abnormal PrP that accumulates during natural and experimental sheep scrapie, as reported previously (Jeffrey et al., 2001a, 2003; González et al., 2003). These results suggest that it may be possible to base European-wide surveillance for natural sheep BSE on the truncation patterns of abnormal PrP.

Genotype-associated variation in the distribution and magnitude (proportion of positive lymphoid follicles and intensity of immunolabelling) of PrP<sup>D</sup> accumulation in LRS tissues was observed in sheep infected with the BSE agent by the IC route. All of these parameters were greater in animals of the PrP ARQ/ARQ genotype than in VRQ/VRQ sheep. The most conspicuous genotype effect, however, was the absence of any LRS involvement in ARR/ARR sheep, which is in agreement with a previous report (Houston et al., 2003). Although peripheral replication is probably irrelevant to incubation period following IC challenge, there appears to be a genotype-related inverse relationship between LRS involvement and incubation period. Thus, an increased length of incubation period corresponds to a lower frequency and magnitude of PrP<sup>D</sup> accumulation in LRS tissues (Fig. 5). The incubation period of sheep TSEs is often taken as a measure of susceptibility, and a greater susceptibility of ARQ/ARQ sheep to BSE infection has also been suggested in previous IC or oral experiments, although VRQ homozygotes were

![Fig. 4. Western blots of PrP<sup>RES</sup> in sheep brain. Representative examples of BSE in each genotype are presented. N, Natural Suffolk scrapie; MM, molecular mass standards; 1–8, experimental BSE, IC challenges (lanes 1 and 2, ARR/ARR; 3 and 4, ARQ/ARQ, 5 and 6, VRQ/VRQ, 7 and 8, ARQ/ARQ). Sheep breeds: Suffolk, lane 1; Cheviot, lanes 2–8. (a) PrP<sup>RES</sup>, PK-treated, mAb 6H4; (b) PrP<sup>RES</sup>, PNGase-treated, mAb 6H4; (c) PrP<sup>RES</sup>, PK-treated, mAb P4.](image)

![Fig. 5. Graph showing relationship between genotype, route of inoculation, mean percentage of positive follicles in three lymphoid tissues and mean incubation period. Open symbols are from orally (AHQ/AHQ) or IV (ARQ/ARQ and ARQ/AHQ)-challenged sheep. Filled symbols are from IC-challenged sheep. Linear regression lines indicate a strong correlation between the incubation period and the percentage of PrP<sup>D</sup>-positive follicles in lymphoid tissues. For tonsil, $r = -0.85$; for MsLN, $r = -0.84$; for spleen, $r = -0.90$.](image)
not represented in either of these studies (Foster et al., 1993, 2001a). All orally or IV-inoculated sheep homozygous for glutamine at codon 171 had levels of peripheral PrP\textsuperscript{d} accumulation approximately similar to those of IC-challenged sheep of the ARQ/ARQ genotype. When the incubation periods and levels of peripheral tissue involvement are viewed together (Fig. 5), this suggests that ARQ/ARQ, AHQ/AHQ and AHQ/ARQ sheep have similar susceptibilities to infection. Alternatively, the PrP of such sheep would have similar efficiencies of conversion from normal to abnormal forms and the accumulation of these would therefore cause disease in a similar time course. In contrast, sheep bearing the VRQ allele would be able to generate less abnormal PrP, as a result of a lower ability either to replicate infectivity or to convert PrP\textsuperscript{c} to PrP\textsuperscript{d}, than sheep of the ARQ/ARQ, AHQ/AHQ and AHQ/ARQ genotypes. Finally, ARR/ARR sheep would be the least able to replicate infectivity in LRS tissues or to support PrP\textsuperscript{c}-to-PrP\textsuperscript{d} conversion.

The presence of abnormal forms of PrP, as determined either by IHC or by Western blotting, is generally taken as a surrogate for the presence of infectivity (Prusiner, 1999). Previous reports on natural scrapie and ovine BSE induced experimentally by the oral route describe ubiquitous PrP\textsuperscript{d}. This variation might be related to cell-driven differences in PrP\textsuperscript{d} conformation, to cell-specific variation in the enzymic content or to other biochemical conditions within lysosomes. These same patterns of between-cell variation of truncation were found in the present study, with the putative shortest fragment of intracellular BSE PrP\textsuperscript{d} being found in TBM's and the longest in neurons, with glia occupying an intermediate position. As these patterns were common to all PrP genotypes, the present study shows that, in ovine BSE, PrP\textsuperscript{d} processing and the resultant variable fragment sizes of PrP\textsuperscript{d} in different cell types are not due to any conformational changes in PrP derived from amino acid substitutions at codons 136, 154 and 171.

In conclusion, this study shows that the antibody-labelling patterns of intracellular PrP\textsuperscript{d} are the same in sheep of different genotypes infected with BSE by different routes and can be differentiated readily from those of all natural sheep scrapie sources so far examined. Wide-scale testing for sheep BSE based on proteinase-digested PrP\textsuperscript{res} fragment sizes may therefore be feasible. In addition, these findings provide support for earlier studies and further suggest that sheep homozygous for alanine at codon 136 and glutamine codon 171 are more susceptible to BSE-agent infection than are other sheep genotypes.

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