Monitoring for bovine spongiform encephalopathy in sheep in Great Britain, 1998–2004

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INTRODUCTION

Transmissible spongiform encephalopathies (TSEs) are a group of fatal, neurodegenerative diseases that affect humans and animals. TSEs of humans include Creutzfeldt–Jakob disease (CJD) and the new variant of CJD (vCJD) (Will et al., 1996). TSEs of animals include scrapie in sheep and goats, endemic in many countries for over 250 years (Detwiler, 1992), and bovine spongiform encephalopathy (BSE), first discovered in 1986 in the UK (Wells et al., 1987).

Although several lines of scientific evidence indicate that BSE has been transmitted to humans in the form of vCJD (Collinge et al., 1996; Lasmézas et al., 1996, 2001; Will et al., 1996; Bruce et al., 1997; Hill et al., 1997), scrapie has never been associated with human forms of the disease. However, it has been reported that BSE has been transmitted experimentally to sheep by oral challenge with as little as 0.5 g bovine brain material (Foster et al., 1993). The UK Spongiform Encephalopathy Advisory Committee considered it likely that, in the UK during the 1980s, BSE-contaminated meat and bone meal were fed to sheep and it was therefore possible that BSE may have been transmitted to the national sheep flock in Great Britain.

Diagnosis of sheep TSEs is accomplished by detection of disease-associated prion protein (PrPd), a term used when processing of tissue samples does not involve protease digestion, such as immunohistochemistry (IHC). PrPd is partially protease-resistant and is usually termed PrPres when detected by Western blotting (WB) techniques (Towbin et al., 1979), which do use protease digestion. PrPres is visualized as three protein bands or glycoforms, which relate to diglycosylated, monoglycosylated and unglycosylated regions of the abnormal protein as detected
with an appropriate antibody, produced against a particular amino acid sequence of the protein (Prusiner et al., 1984; Hope et al., 1988).

Our previous WB studies (Stack et al., 2002), using a modified version of the commercially available Prionics-Check WESTERN technique (Prionics Ag), have shown differences in the electrophoretic mobility of the protein bands, in particular the molecular mass of the unglycosylated protein band of PrP\(^{\text{res}}\), which is higher for scrapie samples than for samples from sheep infected experimentally with BSE. Discrimination was also possible by parallel testing using two specific mAbs. The first, mAb 6H4, is raised to human PrP aa 144–152 (Korth et al., 1997) and detects PrP\(^{\text{res}}\) in cattle and sheep; the second, mAb P4, is raised to ovine PrP aa 89–104 (Harmeyer et al., 1998) and is more selective for scrapie PrP\(^{\text{res}}\). The basis for discrimination is the location of the N-terminal cleavage site for proteinase K digestion of PrP\(^{\text{res}}\) between BSE and scrapie. It has been shown, by antibody-blocking techniques and Pepscan analysis, that the N-terminal amino acid sequence WGQGGSH remained intact only in sheep scrapie samples, thus being detected by the mAb P4 (Thuring et al., 2004).

Detection of the disease-associated form of PrP\(^{\text{d}}\) in neural and non-neural tissue sections of sheep scrapie that have been fixed with formalin can be achieved by using IHC techniques (Miller et al., 1993; van Keulen et al., 1996). Our recent IHC studies have shown that infection with different sheep TSE sources gives rise to distinct morphological and cell-associated accumulations of PrP\(^{\text{d}}\) in the brain (the PrP\(^{\text{d}}\) profile) (González et al., 2002). The differences in PrP\(^{\text{d}}\) profile between sheep TSE sources appear to be the result of variations in cell tropism and in PrP processing, which are possibly associated with the changes in PrP\(^{\text{d}}\) conformation and are manifest as differences in intracellular truncation and release to the extracellular space of the abnormal protein (González et al., 2003). The differences in intracellular truncation of PrP\(^{\text{d}}\) are manifest as distinct labelling patterns of specific N-terminal sequences of PrP\(^{\text{d}}\) within phagocytic cells and neurons (Jeffrey et al., 2001). Thus, sheep BSE cases show a marked reduction in intraacellular immunolabelling of amino acid residues 93–99 of PrP\(^{\text{d}}\) when compared with natural and experimental scrapie cases (Martin et al., 2005). Three different N-terminus labelling patterns have been recognized in different cell types of BSE-infected sheep, suggesting that different truncated forms of PrP\(^{\text{d}}\) are formed following infections with this agent (Jeffrey et al., 2003).

The present report shows the results obtained when the WB technique was used to screen 2368 routine scrapie-suspect brain samples, submitted between 1 January 1998 and 31 May 2004. The samples were divided into two groups. The first group comprised brain tissue from scrapie-suspect sheep, submitted for statutory testing between 1 January 1998 and 31 October 2001, which had been archived previously and was therefore tested retrospectively for unusual molecular profiles. The second group comprised fresh brain samples submitted for statutory testing between 1 November 2001 and 31 May 2004 and was tested prospectively, using the discriminatory WB as one of the statutory tests. The IHC discriminatory technique, also using two antibodies, was applied when equivocal WB results arose from either of these sample groups and to a subgroup of 315 scrapie-suspect submissions. The results of these studies also enabled epidemiological interpretation with respect to the likelihood that BSE exists in the national flock.

**METHODS**

**Animals and tissues.** For the retrospective testing, frozen tissue and, where available, formalin-fixed wax blocks of the medulla oblongata at the obex, taken from 1247 scrapie-suspect sheep between 1 January 1998 and 31 October 2001, were retrieved from the archives. Frozen caudal medulla is the brain region of choice for WB testing but, in some cases, this was not available and different areas of brain or spinal cord were used. Where possible, the brain region tested and the presence of any autolysis were recorded. For the prospective testing, samples (n = 1633) from sheep clinically suspected of scrapie were submitted to VLA Weybridge, UK, between 1 November 2001 and 31 May 2004, as formalin-fixed medulla at the obex for histopathological and IHC examinations and as fresh/frozen caudal medulla for the modified WB technique. Prospective samples were not stored for lengthy periods or subjected to freeze–thawing cycles, as was the case with some of the retrospective samples.

**Western immunoblotting.** All samples were tested by the modified WB technique in parallel by using the two mAbs 6H4 (Prionics Ag) and P4 (BioPharm Ltd). In our previous studies (Stack et al., 2002), sheep scrapie PrP\(^{\text{res}}\) was detected by both mAbs, whereas cattle BSE PrP\(^{\text{res}}\) was only detected by mAb 6H4 and experimental BSE in sheep gave a much-reduced signal with mAb P4. The method used and the interpretation of results have been published previously (Stack et al., 2002). For mAb 6H4:mAb P4 ratios (Nonno et al., 2003), the total amount of PrP\(^{\text{res}}\) signal emitted from the diglycosylated, monoglycosylated and unglycosylated protein bands was calculated by means of the volume-analysis tool in Quantity One software (Bio-Rad). By using the volume-analysis tool, total signal intensity inside a defined boundary drawn on the image was measured and the global background was subtracted (volume = sum of the intensities of the pixels within the defined boundary × pixel area; volume units = intensity units × mm\(^2\)). Any variability was reduced by normalizing the ratio obtained in comparison to a scrapie-control sample, whose value was set at 1; the ratio for the unknown samples was then calculated relative to the scrapie-control sample. Any ratio below 2 : 1 (total volume for mAb 6H4 : total volume for mAb P4) was considered to be scrapie and any ratio above this was considered to be suspicious of BSE (Nonno et al., 2003).

For discriminatory WB screening, all retrospective and prospective samples were tested alongside two control samples, one from a cattle BSE case and one from a Suffolk sheep scrapie case with the PrP\(^{\text{ARQ/ARQ}}\) genotype. Both had positive results previously by all three statutory tests and gave a reproducible molecular profile.

**Discriminatory IHC.** Based on the WB results obtained from the retrospective study, 54 sheep in total were selected for further detailed examination by discriminatory IHC, as described below. Twelve of these cases were of particular interest and were from various geographical areas of Great Britain; three came from the same...
flock (two VRQ/VRQ and one VRQ/ARQ) and the other nine were from individual flocks (two VRQ/VRQ, four ARQ/ARQ, one VRQ/ARQ and two of unknown genotype).

From the prospective study, a subgroup of 315 sheep, submitted between 2 April 2003 and 22 December 2003, was examined by discriminative IHC as a control study.

Discriminatory IHC examinations were performed on formalin-fixed, paraffin wax-embedded samples of medulla oblongata taken at the obex. Tissue samples were cut at 4 μm, pre-treated and processed for IHC detection of PrPSc as described previously (González et al., 2002). The two primary PrP antibodies used for BSE/scrapie discrimination were mAbs R145 and P4, as reported elsewhere (Jeffrey et al., 2003); these two mAbs allow discrimination between BSE and scrapie based on the different patterns of intracellular truncation observed in scrapie and sheep BSE. In addition, brain samples from the two sheep selected from the prospective study (samples 1 and 2) were also immunolabelled with 8G8, 505 and 521 PrP antibodies. On each of the IHC runs, three positive brain-tissue samples were used for comparison: CH1641 experimental sheep scrapie, experimental sheep BSE and natural Suffolk sheep scrapie.

**Epidemiology.** Even if all the cases tested prove to be scrapie, the possibility of BSE cannot be eliminated, in which case an upper 95% confidence limit for the proportion of sheep TSE cases that could be BSE, the number of flocks tested was used, as this is a more appropriate, and conservative, sample size.

**RESULTS**

**Western immunoblotting**

**Retrospective study.** For the retrospective study, 1247 samples were retrieved in total, with submission dates between 1 January 1998 and 31 October 2001. These animals had all previously been diagnosed as scrapie-positive cases by statutory tests. Of these, 1196 were positive by the discriminatory WB test and gave uniformity of results indicative of scrapie.

The molecular masses for the three PrPSc protein bands obtained with mAb 6H4 were consistent and mAb P4 signals for these 1196 samples and the sheep scrapie control included in each gel were of an intensity similar to or greater than that detected with mAb 6H4. The cattle BSE sample included in each gel run as a control was visualized by mAb 6H4, but was not detected by using mAb P4. On the basis of these WB data, we conclude that none of this subset of 1196 cases contained PrPSc that showed BSE-like characteristics.

Of the remaining 51 samples, 35 gave a weak signal with mAb 6H4 and it was often not possible with these samples to see the lower, unglycosylated PrPSc band. Low mAb 6H4 signal intensity and an incomplete banding pattern compromise the interpretation of the WB method and, so, these samples could not be classified as either BSE- or scrapie-like. Retesting of samples from other aliquots of stored, frozen tissue from these cases did not improve the interpretation of the blots and poor tissue quality was the likely cause of this limitation. Similar poor quality of sample was the suspected reason why no mAb 6H4 or mAb P4 signal could be seen in eight of the remaining 16 samples. The final eight cases gave increased staining with mAb 6H4, but appeared to have an unglycosylated PrPSc band with an electrophoretic mobility lower than that of the internal gel scrapie-control sample (results not shown). Four of these latter samples gave reduced intensity of staining with mAb P4 and the other four gave a staining intensity similar to or greater than that with mAb 6H4. In five of these cases, the tissue available for the retrospective testing by WB was not the optimum brain region for testing, as four were from the cerebral cortex and one was from spinal cord. All five were also badly autolysed. Brainstem tissue, which is optimal, was available for the other three cases. These three cases were three of the four that gave strong staining with both mAbs P4 and 6H4. These eight problematic cases and the 42 cases in which the WB result was inconclusive or negative were investigated further by using the differential IHC method. Only 50 samples of the 51 could be referred for IHC, because there was no fixed tissue available for one of the samples. A further four samples from the 1196 samples that we deemed to be scrapie were also referred for discriminative IHC after a blinded examination of all retrospective and prospective VLA WB results was carried out by an experienced examiner from an independent laboratory [Agence Francaise de Sécurité Sanitaire des Aliments (AFSSA), Lyon, France]. These four samples were considered to show a slightly lower electrophoretic mobility for the unglycosylated band with mAb 6H4 by the independent examiner. Three of these had no reduction in the signal detected with mAb P4 and one had a slightly weaker mAb P4 reaction.

**Prospective study.** Out of a total of 1633 clinically suspect submissions received between 1 November 2001 and 31 May 2004, 1121 caudal medulla samples were reported as positive for scrapie by the statutory-testing procedures. The immunoblots obtained from 1119 samples contained PrPSc that showed no BSE-like characteristics. The remaining two samples were referred for further investigations and were termed samples 1 and 2. The independent examiner agreed with the unusual nature of these two samples and confirmed the uniform nature of the 1119 samples considered to be typical for scrapie.

When processed with mAb 6H4, sample 1 gave a low molecular mass, particularly for the unglycosylated band, in
comparison to the control samples and a much-reduced reaction with mAb P4. This sample was from a clinical TSE-suspect sheep that was 17 months old, carried the ARQ/ARQ genotype and was submitted in January 2004. Sample 2 was from a 4–5-year-old ARQ/ARQ sheep from the same flock as sample 1. Whilst sample 1 was selected because of a BSE-like WB result, sample 2 was identified from the Scrapie Notification Database (SND) and, although WB analysis of the caudal medulla originally performed at the time of submission in January 2002 gave a typical scrapie profile (molecular masses were similar to those of the scrapie control used on the gel and similar signal densities were obtained with both mAbs), brainstem from this sample was retrieved from the VLA tissue archives and reanalysed. This time, the sample gave a molecular profile very similar to that obtained for sample 1. There was no evidence on the SND that one of these sheep was the offspring of the other.

To further investigate the initial results for samples 1 and 2, they were retested alongside two cattle BSE- and three sheep scrapie-control tissues. The sheep scrapie-positive controls were: scrapie 1, Suffolk Cross, 4 years old with PrP genotype ARQ/ARQ; scrapie 2, Texel, 3 years old, PrP genotype ARH/VRQ; scrapie 3, Texel, 2 years old, PrP genotype ARH/ARH. Brain tissue from the experimental sheep scrapie strain CH1641 (Foster & Dickinson, 1988), which, in previous studies, shows some molecular characteristics of BSE (Hope et al., 1999; Stack et al., 2002), and sheep BSE brain originating from a primary oral-challenge Romney breed sheep with the PrP<sup>ARQ/ARQ</sup> genotype (Stack et al., 2002), were also included for comparison.

When processed with mAb 6H4, sample 1 again gave a consistently low molecular mass, particularly for the lowest unglycosylated protein band, which was similar to those of the cattle BSE positive control, BSE in sheep control and the CH1641 scrapie strain. All showed increased electrophoretic mobility in comparison to the three natural scrapie samples (Fig. 1, left-hand blots). Signal strengths for mAb P4 were also reduced greatly for all three protein bands for these samples in comparison to the natural scrapie controls (Fig. 1, right-hand blots).

When mAb 6H4 was applied to sample 2, it also gave a low molecular mass, particularly for the lowest, unglycosylated protein band, which was again similar to those of the cattle BSE positive control, BSE in sheep control and the CH1641 scrapie strain, and lower than those of the three natural scrapie controls (Fig. 2, left-hand blots). Signal strengths for mAb P4 were reduced for all three bands, but not as much as the reduction found for sample 1 (compare Figs 1 and 2, right-hand blots). Both of these criteria, the lower molecular mass and the reduced signal from mAbP4, are consistent with the results found for the experimental BSE in sheep samples.

A summary of the results for the retrospective and prospective groups of samples that were tested by using the WB technique and those samples referred for discriminatory IHC is shown in Table 1. Any sample that had a high molecular mass (akin to the positive scrapie-control sample) and was positive with both mAbs was considered to be scrapie. All other samples that were unclassifiable by the WB result (as described above) were referred for discriminatory IHC.

The results for mAb 6H4 : mAb P4 ratios for the 12 samples referred for IHC from the retrospective study were all below the cut-off point, which was set at a value of 2, based on the method of Nonno et al. (2003) (results for the 12 samples are not shown). The results for samples 1 and 2 and the controls are shown in Fig. 3. The cut-off point for scrapie samples was again considered to be below 2; anything above would be suspicious of BSE in sheep, according to a previous publication (Nonno et al., 2003). Sample 1 gave a ratio of 8·5 : 1, similar to experimental BSE in sheep (7·5 : 1), and sample 2 was just above the cut-off, at 2·6 : 1.

**Fig. 1.** Discriminatory Western immunoblots of sample 1 (#1, duplicate lanes) plus control samples (single lanes) processed by using mAbs 6H4 and P4. Lane 1 is the Prionics control, which is bovine PrP<sup>c</sup>; lanes labelled 2 are molecular mass markers loaded in three positions on the gel and the 29 kDa marker is arrowed. Lanes 3, 4 and 5 are sheep scrapie samples, lane 6 is the experimentally infected BSE in sheep sample, lanes 7 and 8 are bovine BSE cases and lane 9 is the experimental sheep scrapie strain CH1641.
IHC

Retrospective study. The discriminatory IHC results for the retrospective and prospective testing are summarized in Table 2. Essentially, seven of the 54 cases examined showed no PrP<sup>d</sup> accumulation and were therefore not confirmed as sheep TSE by IHC. Two samples showed evidence of PrP<sup>d</sup> accumulation, but most of the tissue consisted of white matter, precluding accurate appraisal of intraneuronal and extracellular PrP<sup>d</sup> (shown in Table 2 as unsuitable).

All of the remaining 45 cases showed significant accumulation of a wide range of PrP<sup>d</sup> types (González et al., 2002) in one or more neuroanatomical nuclei at the level of the obex when labelled with mAb R145. For each sheep, at sites where intraneuronal or intraglial labelling occurred in the mAb R145-labelled section, paired serial sections labelled for the mAb P4 epitope also showed similar magnitudes of these same labelling patterns. None of these 45 samples, which included the 12 samples that gave low molecular masses by WB (see Table 1), were therefore considered as showing intracellular-truncation patterns of PrP<sup>d</sup> consistent with experimental ovine BSE (Jeffrey et al., 2003).

Prospective study. The PrP<sup>d</sup> IHC control study of 315 suspect samples identified 80 negative samples and 235 positive cases, in agreement with their original statutory diagnostic results (Table 2). By using the R145/P4 combination of PrP antibodies, the PrP<sup>d</sup> patterns of all of the positive cases were considered within the range of natural scrapie. None showed any reduction in the amounts of intraneuronal and intraglial labelling with the mAb P4 antibody when compared with the R145 antibody. Results for samples 1 and 2 are summarized in Table 3. When labelled with mAb R145, samples 1 and 2 showed prominent intraneuronal and intramicroglial PrP<sup>d</sup> accumulation (as did the scrapie, BSE and CH1641 controls) and sample 2 also showed extensive extracellular deposits. Sample 1 differed from BSE control tissues by the lower amount of extracellular PrP<sup>d</sup> labelling with all antibodies. Neither this sample nor the BSE control showed any intra-

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**Table 1. Results using the discriminatory Western immunoblot technique (DWB) with mAbs 6H4 and P4 and numbers screened and referred for discriminatory IHC**

<table>
<thead>
<tr>
<th>Sample group</th>
<th>No. tested</th>
<th>MM of unglycosylated band</th>
<th>mAb 6H4 signal</th>
<th>mAb P4 signal</th>
<th>Conclusion/action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retrospective</td>
<td>1192</td>
<td>High</td>
<td>Strong</td>
<td>Strong</td>
<td>Scrapie</td>
</tr>
<tr>
<td>Retrospective</td>
<td>35</td>
<td>Negative by DWB</td>
<td>Weak</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>Retrospective</td>
<td>8*</td>
<td>Negative by DWB</td>
<td>Negative</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>Retrospective</td>
<td>7</td>
<td>Low</td>
<td>Strong</td>
<td>Strong</td>
<td></td>
</tr>
<tr>
<td>Retrospective</td>
<td>5</td>
<td>Low</td>
<td>Strong</td>
<td>Weak</td>
<td></td>
</tr>
<tr>
<td><strong>Retrospective total</strong></td>
<td><strong>1247</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prospective</td>
<td>1119</td>
<td>High</td>
<td>Strong</td>
<td>Strong</td>
<td>Scrapie</td>
</tr>
<tr>
<td>Prospective sample 1</td>
<td>1†</td>
<td>Low</td>
<td>Strong</td>
<td>Weak</td>
<td></td>
</tr>
<tr>
<td>Prospective sample 2</td>
<td>1†</td>
<td>Low</td>
<td>Strong</td>
<td>Weak</td>
<td></td>
</tr>
<tr>
<td><strong>Prospective total</strong></td>
<td><strong>1121</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Both referred for discriminatory IHC.

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glial PrP<sup>d</sup> or any intraneuronal PrP<sup>d</sup> in the dorsal motor of the vagus or accessory cuneate nuclei. However, intraneuronal PrP<sup>d</sup> was detectable in the olives of sample 1 when labelled with P4, but was absent in the same nuclei of the experimental BSE control with the same antibody. Sample 2 differed from BSE by the intraneuronal and intraglial labelling patterns with mAbs P4 and 521, respectively, at the accessory cuneate and dorsal motor of the vagus nuclei, so that positive immunolabelling was observed for the test sample, but not for the BSE control. However, no specific intracellular immunoreaction was detected with these antibodies in the olives of sample 2, in which respect it did not differ from the BSE control. Therefore, on the basis of the discriminatory IHC results, neither of these two samples was considered to give a BSE-like pattern.

**Epidemiology**

The absence of BSE-like results for the 2147 cases from 450 flocks implies that, at most, 0·66% of sheep TSE cases could be BSE. Assuming that the proportion of sheep TSE cases that could be BSE provides a reasonable estimate for the proportion of sheep TSE infections that could be BSE, it is possible to extrapolate to estimate the maximum number of sheep flocks that could be affected by BSE. An estimate for the proportion of flocks affected by sheep TSEs in 2002 (1%) is available from an anonymous postal survey (Sivam et al., 2003). Because the results of the retrospective and prospective studies cover a 6-year period, it is more appropriate to use only the results for cases reported in 2002 (392 cases from 122 flocks), which yield an estimate for the maximum proportion of BSE-affected flocks in 2002 of 0·02% (i.e. 20 flocks at most).

**DISCUSSION**

Our interpretation of the results from the present techniques on the retrospective and prospective samples that were available to us provides no evidence for the presence of BSE in sheep. For the retrospective study, some interpretational difficulties with tissues were expected, given that brain region and condition of tissue from stored samples

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**Table 2. Discriminatory IHC (DIHC) interpretation of referred cases**

<table>
<thead>
<tr>
<th>Samples referred for IHC</th>
<th>No. tested (DIHC)</th>
<th>Staining of intraneuronal and intramicroglial PrP&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Conclusion/action</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mAb R145</td>
<td>mAb P4</td>
</tr>
<tr>
<td>Control study</td>
<td>235</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Control study</td>
<td>80</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td><strong>Control study total</strong></td>
<td><strong>315</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Retrospective</td>
<td>7</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>Retrospective</td>
<td>2</td>
<td>Unsuitable</td>
<td>Unsuitable</td>
</tr>
<tr>
<td>Retrospective</td>
<td>45</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td><strong>Retrospective total</strong></td>
<td><strong>54</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prospective sample 1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prospective sample 2</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Prospective total</strong></td>
<td><strong>2</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

DIHC results for samples 1 and 2 are shown in detail in Table 3.
may not have been optimal for the WB testing, particularly when the only available frozen tissue was autolysed or from brain regions other than the brainstem. In our previous evaluations of the reproducibility of results and effects of laboratory-induced autolysis on the molecular profile of PrP\textsubscript{res} (using mAb 6H4 only), we found that the molecular masses of PrP\textsubscript{res} in fresh, frozen brain of scrapie cases was consistently higher for fresh tissue than for autolysed material from the same cases. There were small but significant differences, particularly for the unglycosylated protein band, with a \(p\) value of 0.024 (unpublished results). Studies using the discriminatory IHC technique on formalin-fixed, autolysed tissue have suggested a loss of discriminatory potential with the N-terminal antibody mAb P4 (M. Jeffrey, unpublished results). The effect of autolysis of the mAb P4 epitope at the N terminus of PrP\textsubscript{res} has not been evaluated for the discriminatory WB technique, but this peptide sequence is critical for the modified WB method and could be particularly vulnerable to the endogenous proteolysis occurring during the natural autolytic process. Therefore, the interpretation of discriminatory WB results on autolysed tissue should be viewed with caution. However, as formalin-fixed medulla at the obex was available for the majority of inconclusive samples from the WB screening, we could investigate further by using the differential IHC method.

Phenotypic characterization of sheep TSEs is contributed to by the IHC-truncation pattern of intracellular PrP\textsuperscript{d} (Jeffrey et al., 2001, 2003) and by the relative proportion of different morphological PrP\textsuperscript{d} types (González et al., 2002, 2003). Thus, brains of sheep infected experimentally with BSE or CH1641 show a marked reduction in the detection of intraneuronal and intraglial labelling with mAb P4 when compared with antibodies recognizing the C terminus of the PrP molecule, which is irrespective of route of inoculation, genotype or breed of sheep (Jeffrey et al., 2001, 2003; Martin et al., 2005) and whether sheep are infected with primary- or secondary-passage material (unpublished results). CH1641 is differentiated readily from BSE, as intraneuronal PrP\textsuperscript{d} is cleaved at an amino acid segment more towards the C terminus than those of BSE PrP\textsuperscript{d} (Jeffrey et al., 2006) and also has a distinctive PrP\textsuperscript{d} profile (González et al., 2005). Based on these features, none of the 45 cases from the retrospective study that were examined by discriminatory IHC were considered to show intracellular-truncation patterns of PrP\textsuperscript{d} consistent with experimental ovine BSE or CH1641 infections.

For the prospective study, collection of the precise brain region for the WB test (caudal medulla) had been followed and therefore increased the confidence in results. The molecular profiles for samples 1 and 2 found in this prospective study were clearly different from the scrapie controls and shared some similarities with the experimental BSE in sheep control: low molecular mass and a reduced signal with mAb P4. However, a clear match or a partial match with the results found for experimental BSE in sheep samples must be treated with extreme caution, given the lack of knowledge that we have with regard to serial passage of BSE in sheep, natural transmission between species, breed or genotype effects, pathogenesis and, indeed, a full picture of natural scrapie strains.

Sample 2 has been the subject of intensive tissue audits, as the original caudal medulla submission gave a scrapie profile, whilst the brainstem tissue used in follow-up studies of the flock of origin gave some similarities to experimental BSE in sheep. DNA analysis of the homogenates of caudal medulla and brainstem indicated that they were from the same sheep, ruling out simple sample misidentification as a cause of this discrepancy. It would therefore appear that the two brain areas sampled from this animal gave different molecular profiles. This may be because different cell types process abnormal PrP in a different way, as hypothesized by González et al. (2003).

The IHC results gave the most detailed insight into samples 1 and 2. The results indicated differences between neuroanatomical sites in the patterns of intracellular truncation of PrP\textsuperscript{d}, so that whilst some neuron nuclei showed intracellular loss of the mAb P4 epitope, other sites did not. Whilst this topographical variability in intracellular truncation of PrP\textsuperscript{d} has recently been observed for CH1641 infection (Jeffrey et al., 2006), previous studies on BSE or other sheep scrapie cases had indicated a common global pattern of intracytoplasmic labelling in all neuroanatomical nuclei. The patterns of intracytoplasmic truncation in

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**Table 3. Detailed results of discriminatory IHC of samples 1 and 2**

Only the BSE positive-control sample shows a reduction in intraneuronal labelling of two or more points with mAb P4 when compared with mAb R145, and that reduction was observed in all neuron nuclei. DMNV, Dorsal motor nucleus of the vagus; XII, hypoglossal nucleus. Degree of labelling indicated as: −, absent; +/−, trace; +, weak; + +, moderate; + + +, strong.

<table>
<thead>
<tr>
<th>Sample</th>
<th>DMNV R145</th>
<th>DMNV P4</th>
<th>XII R145</th>
<th>XII P4</th>
<th>Cuneate R145</th>
<th>Cuneate P4</th>
<th>Olives R145</th>
<th>Olives P4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>++</td>
<td>+/−</td>
<td>++</td>
<td>+/−</td>
<td>+</td>
<td>+/−</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Sample 2</td>
<td>+</td>
<td>+/−</td>
<td>+++</td>
<td>+/++</td>
<td>+/+++</td>
<td>+</td>
<td>+/+</td>
<td>+/−</td>
</tr>
<tr>
<td>BSE positive control</td>
<td>++</td>
<td>−</td>
<td>++</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>++</td>
<td>−</td>
</tr>
</tbody>
</table>
samples 1 and 2 were therefore different from those of all previous experimental BSE cases challenged by different routes of inoculation and in different genotypes (Martin et al., 2005).

However, from an epidemiological point of view, the possibility of BSE in the sheep population cannot be excluded completely. An earlier study estimated the maximum proportion of sheep TSE cases that could be BSE to be 5%, based on the results of strain typing in mice (Gravenor et al., 2003), which is much higher than our estimate of 0-66% based on molecular data. The differences between the estimates for the two studies reflect the differences in sample sizes: the strain-typing results were derived from 205 samples drawn from 65 flocks, compared with 2147 samples drawn from 450 flocks for the retrospective and prospective studies. The outcome of this interpretation of the molecular data is that predictions of the likelihood that BSE is present in the national flock, and represents a certain percentage of cases suspected of being due to scrapie, have been revised downwards substantially.

Previously, mathematical models have been used to predict the magnitude of an epidemic of BSE in British sheep under different scenarios (Ferguson et al., 2002; Kao et al., 2002). Our estimate for the maximum number of BSE-affected flocks is higher than was predicted for a feed-borne epidemic of BSE in sheep, but is consistent with the number of affected flocks predicted for an epidemic initiated by contaminated feed and subsequently sustained by horizontal transmission (Kao et al., 2002). Moreover, our estimate for the maximum proportion of BSE-affected flocks is consistent with the predictions for an epidemic of BSE in sheep in which transmission was not self-sustaining either within or between flocks, but not with predictions for two other scenarios in which transmission was self-sustaining either within or between flocks (Ferguson et al., 2002).

There are, of course, some reservations concerning our present approach to search for BSE in sheep: (i) it is not yet possible to validate the molecular methods robustly other than by limited ring trial, i.e. against other tests that use the same principle for discrimination; (ii) the shortage of test material produced in a wide range of genotypes and after multiple subpassages; (iii) it is potentially compromised if sample quality is itself suboptimal; (iv) the fact that the definition of BSE by bioassay requires further investigation, especially with respect to the confidence that can be placed in incubation period in mice. This is not so important in terms of defining the strain when applying all criteria. Greater care is, however, required in the interpretation of incubation periods, especially if there is pressure to prematurely interpret long incubations as being indicative of the absence of BSE.

We have relied on the discriminatory IHC technique to provide results on the problematic samples arising from the WB screening and it is clear that there were some samples in the retrospective group that were equivocal because the initial positive statutory results could not be repeated with either test (Table 2, nine samples in total). We were unable to perform mouse bioassay on these, due to the poor condition of the sample or the fact that there was not enough remaining tissue. Samples 1 and 2 from the prospective group have been submitted for bioassay and we await the results. If either, or both, samples 1 and 2 result(s) in a BSE-like signature in mice from the bioassay, the value of the discriminatory IHC technique will, of course, be decreased. However, following the principles used in this report, there has been a description of the putative case of BSE in a goat in France that was defined by Western immunoblotting followed by mouse bioassay, in the absence of any fixed tissue for IHC investigations (Eloit et al., 2005).

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REFERENCES


