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Discrimination between Scrapie and Bovine Spongiform Encephalopathy in Sheep by Molecular Size, Immunoreactivity, and Glycoprofile of Prion Protein

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A procedure for discrimination between scrapie and bovine spongiform encephalopathy (BSE) in sheep is of importance for establishing whether BSE has entered the sheep population. Since BSE has not yet been found in sheep at the farm level, such discrimination procedures can be developed only with experimental sheep BSE. Two distinctive molecular features of the prion protein (PrP)—molecular size and glycosylation profile—in proteinase K digests of brain stem tissue from sheep were used here; upon Western blotting, these features led to an unequivocal discrimination among natural scrapie, experimental scrapie, and experimental BSE. The higher electrophoretic mobility of PrP in sheep BSE could be best observed after deglycosylation treatment with *N*-glycosidase F. A simpler method for confirmation of this size difference involved comparison of the ratios for the binding of two monoclonal antibodies: P4 and 66.94b4. Based on epitope mapping studies with P4 and peptides, it appeared that N-terminal amino acid sequence WGQGGSH was intact only in sheep scrapie digests. Another feature typical for PrP in sheep BSE was the large fraction of diglycosylated PrP (70% or more). These data were obtained for a large group of positive sheep, consisting of 7 sheep with experimental BSE infection (genotypes: six ARQ/ARQ and one AHQ/AHQ), 48 sheep naturally infected with scrapie (six different genotypes), and 3 sheep with primary experimental scrapie infection. Routine tests of slaughter material serve well for the initial detection of both BSE and scrapie. With Western blotting as a rapid follow-up test, a 66.94b4/P4 antibody binding ratio above 1.5 is a practical indicator for serious suspicion of BSE infection in sheep.

Transmissible spongiform encephalopathies (TSEs), such as bovine spongiform encephalopathy (BSE) in cattle and scrapie in sheep, represent a unique class of infectious diseases for which the mechanism of infection is mainly unknown. Infections are accompanied by the accumulation of an altered form (PrP^{Sc}) of the host prion protein (PrP^{C}), a glycoprotein that is expressed predominantly in neuronal tissues. The unusual properties of the agent have led to the concept that it consists only of protein (29). The most important hallmark is the partial resistance of PrP^{Sc} to degradation by endoproteinases, such as proteinase K (PK). Depending on host species and TSE strain, PK removes 55 to 70 residues from the N-terminal domain of PrP^{Sc}, yielding a distinct product, PrP^{res}, which consists mainly of three glycoforms: aglycosyl, monoglycosyl, and diglycosyl fractions (5, 19, 27, 28, 30). The relative concentrations of these three glycoforms (glycosylation profile) are used as biochemical targets for discriminating infecting TSE agents (12, 18).

The risk that the agent causing BSE may have entered the sheep population has serious implications for human health following compelling evidence that BSE in cattle and a variant form of Creutzfeldt-Jakob disease in humans share a common source of agent (3, 9, 18, 26, 43). For reasons of consumer safety as well as disease control at the farm level, it is of eminent interest that reliable tests be developed for the identification of sheep BSE. These tests should also be cost-effective, fast enough for screening at the slaughter line, and applicable for flock certification. Since BSE has not yet been found in sheep at the farm level, such procedures can be developed only with experimental sheep BSE.

Promising results for discriminating sheep scrapie and experimental sheep BSE in the clinical phase of the disease have been obtained with Western blotting, an effective technique for detection of the three glycoforms of PrP^{res} with PrP-specific antibodies. The molecular size of the aglycosyl fraction of PrP^{res} from the brain is one of the targets for discriminating between the two types of infection but yields partly conflicting results. Most Western blotting studies of sheep brain have reported a larger size for the aglycosyl fraction of PrP^{res} in sheep with natural scrapie than in sheep with experimental BSE (1, 17, 22, 35), while one study showed contradictory examples of either smaller or larger sizes for the aglycosyl

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TABLE 1. Sheep used in this study

Group of sheep	No. of sheep	Genotype	Sheep breed[a]	TSE inoculum[b]	Disease status at tissue collection
Experimentally infected (oral route)	4	ARQ/ARQ	Texel or Tex.cr.	Cow BSE	Clinical signs
	2	ARQ/ARQ	S.C. Cheviot	Cow BSE[c]	Clinical signs
	1	AHQ/AHQ	S.C. Cheviot	Cow BSE[c]	Clinical signs
	3	VRQ/VRQ	Cheviot	Sheep scrapie[d]	Clinical signs
Naturally infected with scrapie	5	ARQ/ARQ	Texel or Tex.cr.		Clinical signs
	20	VRQ/ARH	Texel or Tex.cr.		Clinical signs
	10	VRQ/ARQ	Texel or Tex.cr.		Clinical signs
	4	VRQ/ARR	Texel or Tex.cr.		Clinical signs
	1	VRQ/ARR	Flemish		Clinical signs
	7	VRQ/VRQ	Texel or Tex.cr.		Clinical signs
	1	ARQ/ARH	Texel or Tex.cr.		Healthy, preclinical signs
Negative control	2	ARQ/ARQ	Texel or Tex.cr.		Unknown
	1	VRQ/ARR	Texel or Tex.cr.		Clinically suspect
	1	ARH/ARR	Texel or Tex.cr.		Clinically suspect

[a] Tex.cr., Texel cross; S.C. Cheviot, South Country Cheviot.
[b] All inocula originated from the United Kingdom.
[c] BSE-infected cattle brain pool different from that used for Texel or Texel cross sheep.
[d] Natural sheep scrapie brain pool from 17 sheep.

fraction of PrP^{res} in natural scrapie cases (20). Unfortunately, the aglycosyl fraction has the lowest concentration of the three glycoforms of PrP^{res}, making this approach less attractive for routine diagnosis, unless an additional deglycosylation treatment, e.g., with *N*-glycosidase F (PNGaseF), can yield a more homogeneous discrimination based on size differences. Other features used for discrimination in Western blotting are the type of PrP-specific antibodies and the glycosylation profile of PrP^{res}. Stack et al. (35) reported in a limited study that monoclonal antibody P4 has an affinity for PrP^{res} in natural sheep scrapie but hardly reacts with that in experimental sheep BSE, while monoclonal antibody 6H4 does clearly bind PrP^{res} in both kind of infections. Furthermore, a higher relative concentration of diglycosylated PrP^{res} in BSE-infected sheep than in sheep with natural scrapie infection has been reported (35), although in other reports the concentrations of diglycosylated PrP^{res} were similar for the two disease entities (1, 17).

Scrapie in sheep is much more heterogeneous than BSE in cattle. While for scrapie over 10 different strains have been identified in mouse bioassays, BSE in cattle has until now yielded only 1 strain (8). Also, while in cattle no genetic linkage has been found yet, the susceptibility of sheep to scrapie has been found to be genetically linked to distinct polymorphisms in the PrP gene, notably, in codons 136 (A or V), 154 (R or H), and 171 (Q, R, or H), with ARQ ($A_{136}R_{154}Q_{171}$) being the phylogenetic wild type (4, 21). Arginine at codon 171 is linked to very low susceptibility, while valine at codon 136 is linked to enhanced susceptibility. When studying TSEs in sheep, one should take into consideration factors such as PrP genotype, breed, route, and origin of the infection (6).

The objective of this study was to develop a practical method for Western blotting discrimination between experimental sheep BSE and natural scrapie. This method was used for PK-digested brain stem tissue from a large group of affected sheep, consisting of 7 that were orally infected with BSE (PrP genotypes: six ARQ/ARQ and one AHQ/AHQ) and 48 that had natural scrapie infection (six different genotypes). In addition, three sheep orally infected with unpassaged sheep scrapie, a goat with natural scrapie, and two bovine BSE samples were investigated. Deglycosylation with PNGaseF, epitope mapping studies with different PrP-specific antibodies, comparative analysis of the ratios for the binding to PrP^{res} of monoclonal antibody P4 and novel monoclonal antibody 66.94b4, and determination of the glycosylation profile were explored.

MATERIALS AND METHODS

Animals and tissues. The sheep used in this study are described in Table 1. TSE status was confirmed by histopathologic and immunohistochemical (IHC) analyses. Four homozygous sheep (genotype: ARQ/ARQ) were orally infected with a pool of BSE-infected cattle brain as follows. Texel or Texel cross ewes were maintained and mated in a closed environment where scrapie had not occurred in the past 5 years. Offspring were weaned and transferred to an experimental pathogen class 3 unit at 4 to 5 months of age. Three weeks later, these lambs were orally dosed with 25 ml of a 20% (wt/vol) brain stem homogenate by using disposable syringes. The brain stem homogenate was prepared from approximately 50 brain stems originating from confirmed BSE-infected British cattle. Samples of the same brain stem homogenate pool were used for transmission experiments with Romney sheep (23, 24, 35). The BSE-infected sheep developed clinical signs (ataxia, tremors at the head, and a positive nibbling reflex) at 20, 21, 23, and 24 months after oral dosing. These sheep were euthanatized within 14 days after the onset of clinical signs.

Sheep with natural scrapie ($n = 48$) originated from 13 farms randomly distributed throughout The Netherlands; 11 sheep were from a single farm, and 18 were from an open flock at the Central Institute for Animal Disease Control, Lelystad, The Netherlands. One sheep (ARQ/ARH) without clinical signs or brain pathology was diagnosed with preclinical scrapie by IHC analysis of tonsillar tissue (34, 38, 40). Four sheep which were confirmed to be negative by testing of tonsillar and brain tissues were included. The breed of the animals was Texel or Texel cross, except for one, which was a Flemish breed.

All animals were euthanatized between January 1999 and February 2002. When known, ages varied between 15 and 108 months. Euthanasia by exsanguination was carried out after anesthesia was applied by intravenous injection with sodium pentobarbital (Nembutal; Ceva Sante Animale BV, Libourne, France). Blood samples were collected for genotyping. At necropsy, the brain was dissected sagitally approximately 3 mm from the medial line. For histologic analysis, the larger part of the brain and the medulla oblongata at the level of the obex were fixed in phosphate-buffered formalin (10%). The smaller part of the brain and the brain stem were frozen to below −20°C and used to prepare homogenates.

Brain stem samples from three BSE-infected sheep (two with genotype ARQ/ARQ and one with genotype AHQ/AHQ) were obtained from the Veterinary

Laboratories Agency—Weybridge; they had been generated in South Country Cheviot sheep (13). These animals had been infected through the oral route with a brain pool (composed of six cow brains) different from that used above but similar to those used in other described cases (17, 20; N. Hunter and J. Foster, personal communication). Brain samples—partly from the thalamus and partly from the basal nuclear region—were also obtained from three sheep experimentally infected with scrapie (genotype VRQ/VRQ Cheviot sheep bred in isolation in the United Kingdom from New Zealand-born ewes). These animals had been orally infected with 1 g of a brain pool composed of whole brains from 17 scrapie-infected sheep either homozygous or heterozygous for the VRQ and ARQ alleles.

One Dutch cow with clinical signs of BSE and a goat with scrapie, both confirmed to be positive by IHC analysis and Western blotting, and the challenge BSE inoculum itself were also included in the study.

Diagnostic confirmation. Confirmation of the TSE status for all animals was performed by histopathologic analysis to reveal spongiform lesions and IHC analysis with polyclonal antibodies to ovine PrP sequences from positions 94 to 105 and positions 223 to 234 for sheep and cattle, respectively, to prove the presence of PrP deposits (39, 42). In addition, the TSE status for the sheep, goat, and cow was further evaluated by using a rapid test, routinely used for the diagnosis of BSE and scrapie, with monoclonal antibody 6H4 (Prionics Check; Prionics A.G., Zürich, Switzerland) (25, 33).

Special reagents. PNGaseF either was obtained as a recombinant product from *Escherichia coli* (1,000 U/ml, 25,000 U/mg; Roche Diagnostics, Mannheim, Germany) or was produced from *Flavobacterium meningosepticum* (500,000 U/ml, 1,800,000 U/mg; New England Biolabs, Beverley, Mass.).

Genotyping. Blood from sheep was used for PrP genotyping at least for PrP codons 131, 154, and 171 (7). Because samples were collected over a period of several years, a variety of genotyping techniques were used (denaturing gradient gel electrophoresis, TaqMan analysis, and/or sequencing) (7; A. Bossers, unpublished data). The DNA sequence of PrP in animals that were experimentally infected with BSE was fully determined by sequencing. In addition to the techniques mentioned, samples from all natural scrapie cases were genotyped again by using a novel method (Pyrosequencing AB, Uppsala, Sweden) that approximately reveals mutations between codons 135 and 139, 152 and 156, and 170 and 173 (32; Bossers, unpublished).

Tissue treatments. All activities were carried out in a pathogen class 3 facility in agreement with European Union directives and the guidelines set out by the Spongiform Encephalopathy Advisory Committee in the United Kingdom.

For experiments with deglycosylation treatments, procedures for homogenization, digestion, and detection were as follows. Tissue homogenates (10%) were prepared by homogenizing 0.3 to 0.6 g of brain stem material cut from within 3 cm of the obex region at 30,000 rpm for 60 s in extraction solution (0.01 M Tris-HCl– 0.15 M NaCl– 5 mM EDTA [pH 8.0], 1% [wt/vol] Triton X-100, 0.5% [wt/vol] sodium deoxycholate) by using an Omni International TH mixer with a disposable probe. Digestion of 200 μl of homogenates was carried out at 37°C for 1 h by the addition of 20 μl of PK (0.55 mg/ml; E. Merck AG, Darmstadt, Germany) in phosphate-buffered saline (PBS). Digestion was stopped by the addition of 20 μl of 125 mM Pefabloc SC (Roche). For further deglycosylation, 120 μl of this digest was further denatured by adding 60 μl of extraction solution and 18 μl of denaturing solution (5% sodium dodecyl sulfate [SDS]– 10% β-mercaptoethanol in 20 mM Tris-HCl– 150 mM NaCl– 2 mM EDTA [pH 7.5]) and then heating the mixture for 10 min at 95°C. From this mixture, 100 μl was taken and combined with 125 μl of PBS and 25 μl of 10% Triton X-100. Deglycosylation was carried out for 16 h at 37°C by the addition of 3 U of PNGaseF (1 U/μl; Roche). Before application to gels, the PNGaseF-treated sample (PNGaseF digest) and 120 μl of the PK-treated sample (PK digest) were precipitated with 1 ml of cold (−20°C) methanol and centrifuged in a Microfuge for 10 min at 16,000 x *g*. Pellets were dissolved by careful trituration with 100 μl of sample buffer (0.25 M Tris-HCl [pH 8.5], 2% lithium dodecyl sulfate, 2% β-mercaptoethanol, 0.4 mM EDTA, 0.02% bromphenol blue, 10% glycerol). The samples were heated for 5 min at 96°C and used directly for SDS-polyacrylamide gel electrophoresis (PAGE) (12% bis-Tris gels; NuPAGE; Invitrogen, Breda, The Netherlands).

PrP-specific antibodies. The primary antibodies used were rabbit polyclonal antibody R521 and mouse monoclonal antibodies P4, 6H4, and 66.94b4 (14, 16, 25, 39). Monoclonal antibody 66.94b4 has not been reported before and was prepared by immunizing PrP knockout mice (10) (kindly provided by C. Weissmann, Zürich, Switzerland) with recombinant human PrP (produced in *E. coli* and kindly provided by T. Sklaviadis, Thessaloniki, Greece) and recombinant bovine PrP (Prionics). The antibody was found to be specific for intact PrP^{C} and PrP^{res}; however, it did not bind 15-mer peptides of PrP sequences from various animal species, as assessed by Pepscan analysis (see below). The linear epitope specificities of the other antibodies, including 6H4, could be confirmed.

SDS-PAGE, Western blotting, and 66.94b4/P4 antibody binding ratios. SDS-PAGE under reducing conditions was performed with 10-well precast 12% NuPAGE gels (NuPAGE gel electrophoresis system; Invitrogen). The molecular weight markers used were bovine serum albumin, chicken egg albumin, carbonic anhydrase, and β-lactalbumin (Sigma, St. Louis, Mo.). The sample volume applied was usually 10 μl, representing 400 to 1,000 μg of tissue equivalents. Electrotransfer to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, Mass.) and immunostaining were performed according to established procedures (11, 37). The primary antibodies used were mouse monoclonal antibodies 6H4, P4, and 66.94b4 at 0.2 μg/ml and rabbit polyclonal antibody R521 at 1/2,000 in 50 mM Tris-HCl– 0.15 M NaCl– 2.5 mM KCl–0.05% Tween 20–5% skim milk protein (antibody incubation solution). The secondary antibodies used were rabbit anti-mouse immunoglobulin G (IgG; Dako, Glostrup, Denmark) and goat anti-rabbit IgG (Zymed Laboratories, San Francisco, Calif.), both conjugated to alkaline phosphatase. The signals were developed with CDP-Star by following the supplier's instructions (Tropix, Bedford, Mass.) and were recorded on photographic film (Hyperfilm ECL; Amersham, Buckinghamshire, United Kingdom). The densities of the bands were recorded from the film with an Agfa Duoscan T200XL scanner, further processed with GelPro software (MediaCybernetics, Silver Spring, Md.), and expressed as integrated optical densities. To calculate 66.94b4/P4 antibody binding ratios, integrated optical densities obtained for both antibodies in the same experiment and from films with similar exposure times were used. Molecular weights were determined according to a method described previously (41).

Pepscan analysis. To detect the linear epitope specificities of the antibodies used, Pepscan analysis of solid-phase synthetic peptides bound as described previously was performed in an enzyme-linked immunosorbent assay-like setup (14, 15). Complete sets of overlapping 15-mer peptides which covered the amino acid sequences of bovine PrP and ovine PrP (respective GenBank accession numbers AJ000739 and X55882) were synthesized. A site was considered positive (i.e., antigenic) when the absorbance values of two or more consecutive peptides were at least three times the background. The background was calculated as the mean absorbance value measured for 20 consecutive peptides with low levels of reaction, where the standard deviation (SD) was ≤20% the mean absorbance value.

Peptide synthesis. Peptides with acetylated and amidated termini were synthesized by using 9-fluorenylmethoxy carbonyl chemistry as previously described (39). The products used were checked for identities by molecular mass spectrometry and were brought to over 80% purity by high-performance liquid chromatography, as evaluated by photospectroscopy at 215 nm. Sequences were based on that of ovine PrP. The peptides used were ovPrP89– 107, ovPrP94– 105, ovPrP109– 136^{Ala136}, ovPrP126– 143^{Ala136}, and ovPrP223– 234.

Antibody blocking studies. For blocking experiments, polyclonal antibody R521 (diluted 1/100 in PBS– 0.02% Tween 80) and murine monoclonal antibody P4 (at 14 μg/ml) were incubated overnight with 1 mg of peptide/ml at 4°C. Subsequently, the mixtures were further diluted in antibody incubation solution to a 1/2,000 dilution and to 0.2 μg of mouse IgG/ml, respectively.

Statistical analyses. One-way analyses of variance were carried out to establish whether variations between groups of data were greater than expected; if so, subsequent differences between pairs of groups were considered significant if the probability of a difference was <0.05 in multiple-comparisons tests either according to the Student-Newman-Keuls test or, if differences among SDs were found to be significant by Bartlett's test, according to the nonparametric Welch test. The software used for these calculations was Instat Statistics from GraphPad Software, San Diego, Calif.

RESULTS

Improving the detection of differences in the migration of PrP^{res} in BSE- and scrapie-infected tissues. Upon routine detection of PrP^{res} in brain stem homogenates from sheep infected with scrapie and BSE, typically three protein bands (PrP triplet) were observed (Fig. 1A). These three bands represent the diglycosyl, monoglycosyl, and aglycosyl moieties. A small but reproducible difference in molecular masses was visible between natural scrapie and experimental BSE for each of the PrP triplet bands. The apparent molecular masses (mean and SD) of the protein moieties in a typical experiment were

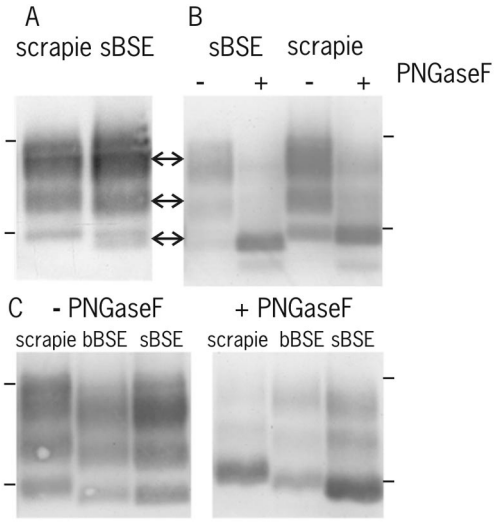

FIG. 1. Migration patterns for PrP^{res} in natural scrapie, experimental sheep BSE, and bovine BSE. (A) Sheep scrapie and sheep BSE (sBSE). The three glycosylation forms of PrP^{res}, diglycosyl, monoglycosyl, and aglycosyl, are indicated by arrows, from top to bottom, respectively. (B) Comparison of migration positions for PrP^{res} triplet bands before and after PNGaseF treatments. The positions of the aglycosyl band before (−) and after (+) deglycosylation by PNGaseF appear to be identical. An additional band below the aglycosyl form is variably present depending on the individual sample. (C) Differences in the migration of the PrP^{res} triplet in sheep scrapie, bovine BSE (bBSE), and sBSE without (−) and with (+) PNGaseF treatments. Molecular mass markers were 29 and 18.4 kDa, and their migration positions are indicated by the upper and lower bars, respectively, beside the panels. The procedures used were the Prionics Check method (antibody 6H4) (A) and the methods designed for tissue treatment and Western blotting for this study (antibody 66.94b4) (B and C). Applied tissue equivalents were 430 μg (A) and 500 μg (with PNGaseF) and 1,000 μg (without PNGaseF) (B and C).

26.3 ± 0.5, 21.4 ± 0.2, and 18.1 ± 0.3 kDa for PrP^{res} in scrapie-infected sheep ($n = 3$) and 25.5 ± 0.5, 20.1 ± 0.3, and 16.5 ± 0.2 kDa in BSE-infected sheep ($n = 4$). As is typical for PrP^{res}, the two glycosyl moieties appeared very diffuse, and the aglycosyl form was often faint. However, the aglycosyl band exhibited the sharpest profile and the largest visible migrational difference between scrapie- and BSE-infected samples. For practical diagnostic reasons, we tried to concentrate all PrP^{res} material in one band by enzymatic deglycosylation. Upon deglycosylation with PNGaseF, which cleaves the N-glycosidic bond between a complete carbohydrate moiety and peptidyl asparagine, most of the diglycosylated PrP^{res} and monoglycosylated PrP^{res} disappeared. Importantly, the migration position of the aglycosyl band was not changed detectably by deglycosylation treatment (Fig. 1B). However, the deglycosylation was often not complete. Attempts to improve the extent of deglycosylation with PNGaseF failed. Attempts included increasing the enzyme concentration to up to 20 U per digest, using two sources of enzyme (recombinant from *E. coli* or authentic from the host, *F. meningosepticum*), adding fresh aliquots of enzyme at different intervals, and extending the incubation time to up to 72 h (data not shown). Nevertheless, in all samples tested so far, the aglycosyl band became more intense after the PNGaseF treatment, a finding which is advantageous for discrimination between sheep infected with natural scrapie and sheep infected with experimental BSE when migrational differences between these two are used as the sole method of detection.

Application of different antibodies to scrapie- and BSE-infected tissue homogenates. Brain stem samples from BSE-infected sheep were compared with brain stem samples from scrapie-infected sheep by using four different PrP-specific antibodies for material that had been digested with PK and PNGaseF. While PrP-specific antibodies 6H4, P4, 66.94b4, and R521 all bound the PrP triplet bands of PrP^{res} in Western blots, it appeared that antibodies 6H4, 66.94b4, and R521 each revealed the same enhanced migrational differences between samples from BSE-infected sheep and samples from scrapie-infected sheep (Fig. 2, lanes 1 and 2). Monoclonal antibody P4 exhibited two aberrant aspects: first, the antibody hardly bound to PrP in samples from BSE-infected sheep; second, when samples were slightly reactive with P4, the migration positions for the PrP^{res} moieties in sheep BSE were similar to those in sheep scrapie. In addition, antibodies P4 and R521 both showed hardly any affinity for PrP^{res} in bovine BSE (Fig. 2, lanes 3).

Comparisons of natural sheep scrapie, experimental sheep BSE, experimental sheep scrapie, and natural bovine BSE. The differences in PrP migration and antibody binding between sheep scrapie and sheep BSE were observed in all seven orally infected BSE sheep, irrespective of breed (Texel, Texel cross, or Cheviot) or PrP genotype (six ARQ/ARQ and one AHQ/AHQ) (Fig. 3A). The migration of PrP^{res} in samples from Cheviot VRQ/VRQ sheep orally infected with an unpassaged brain pool (from scrapie) was similar to that in samples from sheep with natural scrapie infection (Fig. 3B). Thus, while antibodies 66.94b4, 6H4, and R521 bound PrP^{res} in samples from both scrapie and BSE-infected sheep, P4 only bound well to samples from scrapie-infected sheep.

For bovine BSE, the aglycosyl band of PrP^{res} in brain stem samples exhibited migration intermediate between those for sheep scrapie and sheep BSE; this finding was most evident after deglycosylation (Fig. 1C). This finding was obtained both with the inoculum used for BSE-infected sheep (from the United Kingdom) and for Dutch bovine BSE (data not shown).

Epitope characterization of antibodies P4 and R521 with synthetic peptides. Based on the observations described above, it appears that R521 binds well to PrP^{res} in both natural scrapie and sheep BSE, whereas P4 binds well only to PrP^{res} in natural scrapie (and hardly at all to PrP^{res} in sheep BSE). Since P4 and R521 were elicited against partial overlapping peptide sequences—positions 89 to 104 and positions 94 to 105 of ovine

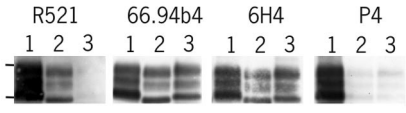

FIG. 2. Four different antibodies tested for three ruminant TSE types. Lanes 1 to 3 contain PK digests of brain stem tissues infected with natural scrapie, sheep BSE, and bovine BSE, respectively. Western blotting of PK/PNGaseF digests was done with the antibodies indicated above the panels. The applied tissue equivalent was 500 μg per lane. Molecular mass markers are as described in the legend to Fig. 1.

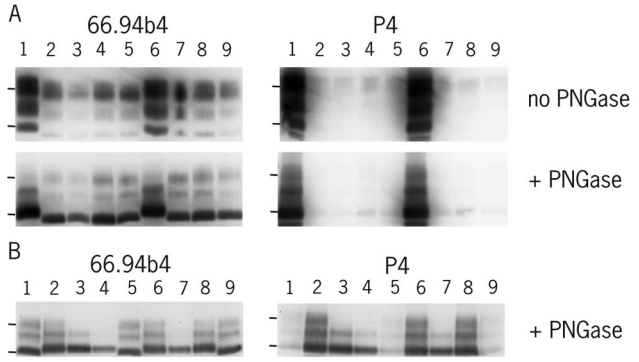

FIG. 3. Different TSEs in sheep analyzed with antibodies 66.94b4 and P4. (A) Analysis of seven experimental sheep BSE cases. Lanes: 1, natural scrapie; 2, BSE1, Texel; 3, BSE2, Texel; 4, BSE1, Cheviot (AHQ/AHQ); 5, BSE2, Cheviot; 6, natural scrapie; 7, BSE3, Cheviot; 8, BSE3, Texel; 9, BSE4, Texel. (B) Analysis of three experimental scrapie cases. Lanes: 1, BSE1, Texel; 2, natural scrapie (VRQ/VRQ); 3, experimental scrapie 1; 4, experimental scrapie 2; 5, BSE1, Texel; 6, natural scrapie (VRQ/VRQ); 7, experimental scrapie 3; 8, natural scrapie (VRQ/VRQ); 9, BSE1, Texel. Table 1 provides details on experimentally infected animals. Applied tissue equivalents were 500 μg (with [+] PNGaseF) and 1,000 μg (without PNGaseF). Molecular mass markers are as described in the legend to Fig. 1.

PrP, respectively—their requirements for binding to ovine PrP were further investigated. Two sets of experiments were undertaken. First, a Pepscan analysis of solid-phase 15-mer peptides based on the ovine PrP sequence revealed that P4 bound peptides around the core sequence 93WGQGGSH99 (Fig. 4). The analysis of R521 was previously described (14): in a Pepscan analysis, this antiserum exhibited binding to three linear sites in the PK-resistant region of PrP^{Sc} (the domain from positions 94 to 105, which was used for immunization, and two sequences in the domain from positions 121 to 146, which was part of PrP^{res}). In a second set of experiments, antibody blocking on Western blots was carried out with synthetic peptides in solution to determine which amino acids in the ovine PrP sequence contributed to binding of the antibodies to immobilized protein. When ovine brain stem material was used, antibody P4 could be blocked fully by the peptide at positions 89 to 107 but not positions 94 to 105. R521 was already fully blocked

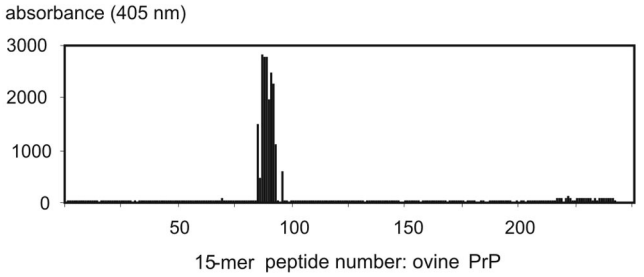

FIG. 4. Epitope mapping of monoclonal antibody P4 by Pepscan analysis with 15-mer solid-phase peptides overlapping the entire amino acid sequence of the ovine PrP gene. Each bar represents the spectrophotometric absorbance signal at one peptide. The peptides recognized by the antibody are numbers 85 to 93 and 95. The common sequence or core sequence between antigenic peptides 85 and 93 in this analysis is 93WGQGGSH99 of ovine PrP. Numbers on the *x* axis represent the N-terminal residue of each 15-mer peptide.

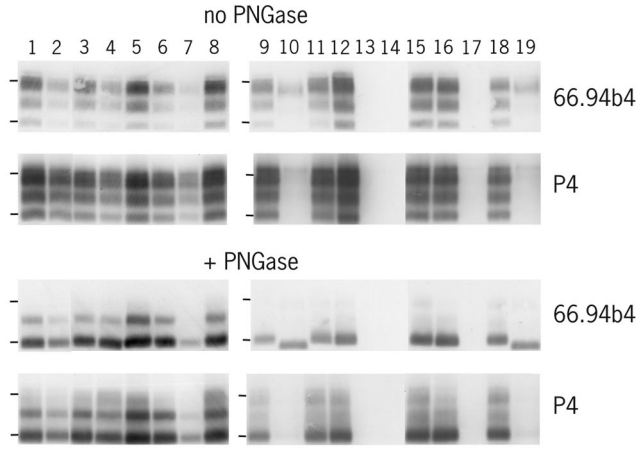

FIG. 5. Comparison of binding of antibody 66.94b4 and antibody P4 in relation to individual scrapie isolates (including that from a goat), a sheep BSE isolate, and different genotypes. Lanes: 1, VRQ/VRQ; 2, VRQ/ARH; 3, VRQ/ARH; 4, ARQ/VRQ; 5, ARQ/ARH; 6, ARQ/ARQ; 7, VRQ/ARH; 8, VRQ/ARH; 9, VRQ/ARH; 10, ARQ/ARQ, BSE2, Texel; 11, VRQ/ARR; 12, ARQ/VRQ; 13, ARQ/ARQ, sheep was scrapie negative but clinically suspect; 14, ARQ/ARH, sheep with preclinical scrapie (clinically negative, tonsil positive, and brain negative); 15, VRQ/ARR; 16, goat with scrapie; 17, ARH/ARR, healthy sheep; 18, ARQ/VRQ; 19, ARQ/ARQ, BSE2, Texel. Half of the samples were subjected to PNGaseF treatment. Applied tissue equivalents were 1,000 μg (without PNGaseF) and 500 μg (with [+] PNGaseF). Molecular mass markers are as described in the legend to Fig. 1.

by the peptide at positions 94 to 105 (data not shown). Appropriate control peptides with sequences from other domains of ovine PrP did not have any visible blocking effect (positions 109 to 136, 126 to 143, and 223 to 234). These results show that the cleavage sites for proteases in PrP^{Sc} are different between sheep scrapie and sheep BSE.

Antibody binding ratios for natural sheep scrapie, experimental sheep scrapie, experimental sheep BSE, and natural goat scrapie. In comparative Western blots with various concentrations of scrapie-infected sheep brain homogenates and antibodies, antibody 66.94b4 exhibited a consistently higher affinity for ovine PrP^{res} than did antibody 6H4 (data not shown). Therefore, 66.94b4 was used for further studies to evaluate whether there were molecular differences between groups of sheep with natural scrapie and sheep with experimental scrapie or BSE. The molecular characteristics of PrP^{res} in our set of 48 sheep with natural scrapie were similar for all six genotypes with respect to the migration positions of the aglycosyl band and the antibody binding properties (66.94b4/P4 ratio) for either the three PrP^{res} bands together or the aglycosyl band after PNGaseF treatment (Fig. 5). There were also no significant differences when the theoretical effect of overrepresentation by samples from a single herd was considered (data not shown). This possibility could be excluded by analysis of two groups of sheep: a herd of 11 scrapie-infected sheep from a single farm (all VRQ/ARH) and a group of 5 scrapie-infected sheep (VRQ/VRQ) from our own flock (data not shown). A sample from a scrapie-infected goat was also tested and did not show any visible difference from samples obtained from sheep with natural scrapie infection for these two properties.

TABLE 2. Antibody binding ratios for PrP^{res} in sheep scrapie, goat scrapie, and sheep BSE[a]

Group	TSE type	Genotype (no. of animals)	Mean ± SD 66.94b4/P4 ratio in:	
			Three PrP^{res} bands	Aglycosyl band after PNGaseF treatment
Natural scrapie	Sheep scrapie	All (48)	0.42 ± 0.22[b]	0.74 ± 0.27[c]
		ARQ/ARQ (5)	0.53 ± 0.12	0.99 ± 0.24
		ARQ/VRQ (10)	0.39 ± 0.18	0.73 ± 0.28
		ARQ/ARH (1)	0.51	1.06
		VRQ/ARH (20)	0.33 ± 0.22	0.64 ± 0.24
		VRQ/VRQ (7)	0.52 ± 0.27	0.71 ± 0.25
		VRQ/ARR (5)	0.53 ± 0.19	0.84 ± 0.23
	Goat scrapie	NA	0.99	1.08
Experimental TSE in sheep	Scrapie	VRQ/VRQ (3)	0.29 ± 0.12	0.62 ± 0.10
	BSE in Texel or Tex.cr.	All (7)	3.29 ± 1.00[d,f]	12.59 ± 7.84[e,f]
		ARQ/ARQ (4)	3.09 ± 1.05	12.34 ± 5.76
	BSE in S.C. Cheviot	ARQ/ARQ (2)	3.35 ± 1.45	6.24 ± 0.09
	BSE in S.C. Cheviot	AHQ/AHQ (1)	3.98	26.30

[a] Ratios were obtained from density recordings as described in Materials and Methods. NA, not applicable; Tex.cr., Texel cross; S.C. Cheviot, South Country Cheviot.
[b] Range, 0.07 to 1.02.
[c] Range, 0.15 to 1.38.
[d] Range, 1.95 to 4.47.
[e] Range, 6.17 to 26.30.
[f] The value is statistically different from that in the following groups: the 48 natural scrapie cases, the 5 multimember-genotype natural scrapie cases, and the experimental scrapie cases ($P < 0.001$).

To further substantiate these observations for the antibody binding properties, density values were calculated for the PrP^{res} bands and transformed to 66.94b4/P4 ratios (Table 2). The 66.94b4/P4 ratios obtained with sheep naturally and experimentally infected with scrapie were similar, both for the three bands together after PK digestion only and for the aglycosyl band after subsequent PNGase F treament. In addition, differences in these ratios among the six genotypes in sheep naturally infected with scrapie were not statistically significant. The ratios in samples from BSE-infected sheep, however, were well above 1.5 and were significantly different from those in samples from scrapie-infected sheep, which all remained below this value (Table 2).

Glycosylation profiles. Glycosylation profiles were analyzed as a possible independent parameter for strain differences (Fig. 6). In our set of samples, the relative concentration of diglycosylated PrP^{res} in the PrP triplet was significantly higher in samples from BSE-infected sheep (mean and SD, 76.9% ± 5.2%; range, 77 to 85%; $n = 7$) than in samples from scrapie-infected sheep (mean and SD, 55.9% ± 6.0%; range, 46 to 71%; $n = 48$) ($P < 0.001$). The concentrations of the monoglycosyl and aglycosyl fractions of PrP^{res} were also significantly lower in BSE-infected sheep than in scrapie-infected sheep ($P < 0.001$). The three samples from sheep experimentally infected with scrapie had the lowest concentration of the diglycosyl form and the highest concentrations of both the monoglycosyl and the aglycosyl forms. There were also no statistically significant differences in glycosyl form concentrations among sheep with different genotypes when the theoretical effect of overrepresentation by samples from a single herd was considered. In the sample from the goat infected with scrapie, the relative concentrations of the three PrP^{res} bands appeared to be similar to those in the samples from sheep naturally infected with scrapie.

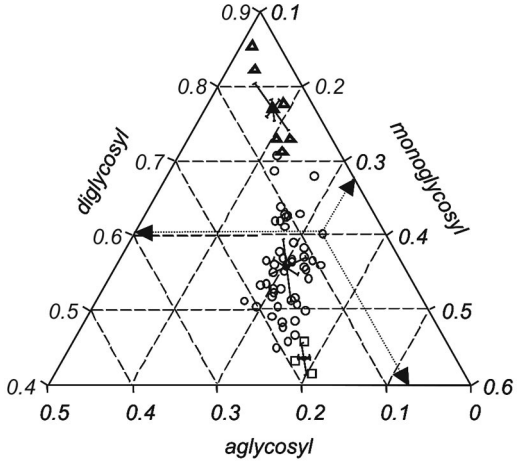

FIG. 6. Triangular plot of individual glycosyl fractions of PrP^{res} after PK digestion for all TSE cases in sheep. In addition to the individual data, the means for all sheep naturally infected with scrapie ($n = 48$), sheep experimentally infected with scrapie ($n = 3$), and sheep experimentally infected with BSE ($n = 7$) are plotted with their means and SDs. Symbols: triangles, sheep BSE; circles, natural sheep scrapie; squares, experimental sheep scrapie. The values for sheep BSE differed significantly ($P < 0.001$) from those for natural sheep scrapie and experimental sheep scrapie. Furthermore, the values for the diglycosyl and monoglycosyl fractions in experimental sheep scrapie deviated significantly from those in natural sheep scrapie ($P < 0.01$). Measurements from density scans were obtained as described in Materials and Methods. Explanation for reading the plot: read the values for the diglycosyl, monoglycosyl, and aglycosyl fractions over lines parallel to the base, the left axis, and the right axis of the triangle, respectively. For each point, the sum of the three values is 1. One individual point has been marked by arrows: its values are 61, 32, and 7% for the diglycosyl, monoglycosyl, and aglycosyl fractions, respectively.

Finally, there was no obvious correlation between a relatively high affinity of P4 for scrapie PrP^{res} (as reflected by a low 66.94b4/P4 antibody binding ratio) and the relative concentration of one of the three PrP^{res} bands (data not shown).

DISCUSSION

This study shows that Western blotting of brain stem homogenates can be used as a rapid means for the diagnosis of a suspected case of BSE in sheep and provides a practical method for obtaining such a diagnosis. This study also extends previous reports which reached basically the same conclusion, but the experiments done here did not produce false-negative diagnoses and were based on a comparison of samples from a large group of sheep infected with scrapie and fully PrP genotyped. Three aspects of PrP^{res} can be used as a basis for suspicion: enhanced electrophoretic migration, very poor binding of antibody P4 to PrP^{res} (relative to that of antibody 66.94b4), and a typical glycoprofile with a high concentration of the diglycosyl fraction. Because the antibody comparison is the most robust, a method for routine diagnosis based on this aspect is suggested.

Of the three molecular mass forms of PK-resistant protein PrP^{res}, the form with the lowest molecular mass—representing the aglycosyl fraction of PrP^{res}—is approximately 1.6 kDa smaller in sheep BSE than in sheep scrapie and is the best form to use if diagnosis is to be based solely on enhanced electrophoretic migration. The reason is that for the two other forms, the differences between BSE and scrapie are smaller than 1.6 kDa and are difficult to assess with the naked eye because of the diffuse nature of the bands caused by the presence of glycosyl groups, which intrinsically convey molecular microheterogeneity to glycoproteins. However, since the aglycosyl moiety represents only 5 to 20% of total PrP^{res}, the aglycosyl band can be very faint or invisible upon routine diagnosis. Indeed, it was found that enzymatic deglycosylation of the other two triplet bands of PrP^{res}—representing the diglycosyl and monoglycosyl fractions—by PNGaseF resulted in an unambiguous diagnosis of BSE when appropriate controls for scrapie were included in the experiment. Although such deglycosylation often is incomplete, the aglycosyl band becomes sufficiently dense in routine diagnostic experiments.

Differential antibody binding is another aspect which can be used for the detection of BSE in sheep. The relative amounts of binding of two antibodies, including one (P4) which does not bind to PrP^{res} in sheep BSE but does in scrapie and one (such as 66.94b4) which binds to PrP^{res} in both sheep BSE and scrapie, were quantitated by densitometric scanning. This analysis led to a cutoff value for the 66.94b4/P4 ratio of approximately 1.5 (at 0.2 μg of IgG/ml for chemoluminescence detection), above which an animal should be considered to be BSE positive, irrespective of whether enzymatic deglycosylation was applied (Table 2). However, visual inspection alone is sufficient to observe this difference in antibody binding (Fig. 3). The very poor binding of antibody P4 to PrP^{res} relative to that of antibody 66.94b4 in sheep BSE confirmed the findings of Stack et al. (35), who compared the binding of antibodies P4 and 6H4 in a limited number of cases. Antibody 6H4 is comparable to antibody 66.94b4 in that it is elicited with recombinant PrP and binds to the PK-resistant domain of PrP^{Sc}. However, analysis for linear epitopes (Pepscan analysis) showed that the specificity of 66.94b4 is profoundly different from that of 6H4; the binding site of 6H4 is a linear one located at amino acids 148 to 156 of ovine PrP (25; this study), but that of 66.94b4 could not be identified. These data indicate a conformation-dependent epitope for 66.94b4. Our study included a larger number of sheep, with respect to both sheep BSE and scrapie. It would be valuable to know whether the material tested by Hope et al. (20) would have yielded more consistent discrimination between natural scrapie and sheep BSE if P4 and 66.94b4 (or another PrP-specific antibody, such as 6H4) had been used.

Another aspect which can be used for BSE detection is a high concentration of the diglycosyl fraction (or a low concentration of the aglycosyl fraction); this concentration was over 70% of total PrP^{res} in the seven BSE-infected sheep studied, while in scrapie-infected sheep, the concentration remained below this value (≤71%). Like us in this study, Stack et al. (35) also noted that the concentration of the diglycosyl fraction of PrP^{res} in two BSE-infected sheep was higher than that in natural scrapie-infected sheep, while Baron et al. (1, 2) and Hill et al. (17) did not observe a clear difference. It is difficult to explain these different observations. They probably do not relate to differences in the region of the brain sampled (36) but may be related more to the type and concentration of antibody used. Furthermore, the data must be considered cautiously, since there is a variation in SDs of 50 to 90% within repeated measurements of individual samples. Not only antibody type but also antibody concentration are of importance for standardizing such strain-specific details. Furthermore, if the glycosylation profile is used to reach a diagnosis, it should be performed with proper controls, such as samples representing natural scrapie and experimental BSE, in the same blot; this requirement is hardly possible for routine laboratories due to the limited availibility of materials for sheep BSE. Nevertheless, our data indicate the same trend as has been observed by others that the concentration of the diglycosyl PrP fraction is relatively high in sheep BSE as well as BSE in other animal species, including humans with a variant form of Creutzfeldt-Jakob disease (12, 18, 31, 35).

The basis for the discrimination between scrapie and BSE in sheep is the location of the N-terminal cleavage site in PrP^{res} by proteolytic digestion. This location was detailed by fine epitope mapping of monoclonal antibody P4 in a Pepscan analysis with solid-phase peptides, blocking studies with synthetic peptides in solution, and a comparison with polyclonal antiserum R521. With antibodies P4 and R521, which bind ovine PrP at sites which overlap each other in the N-terminal domain of PrP^{res}, it was shown that digests of brain stem samples from BSE-infected sheep contained a form of PrP^{res} which lacked amino acids needed for binding by P4. Antibody P4 binds to residues 93 to 99 of ovine PrP, and this binding can be blocked in Western blots for ovine scrapie by a peptide (residues 89 to 107) containing the sequence from residues 93 to 99 but not by an overlapping peptide (residues 94 to 105) lacking Trp93. Since the sequence from residues 93 to 99 is required for optimal binding by P4, it is possible that also amino acids 94 to 99 are absent in PrP^{res} in sheep BSE (Fig. 7). In contrast to antibody P4, antibody R521 binds PrP^{res} in sheep BSE very well. R521 was previously shown to bind broadly to synthetic PrP sequences in this same region by a Pepscan

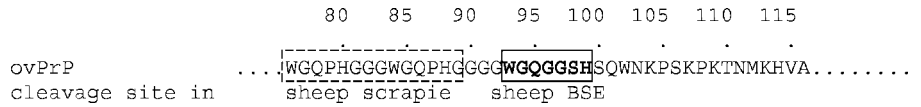

FIG. 7. Depiction of the two regions of cleavage by proteases in ovine PrP^{Sc} for scrapie and BSE. The sequence enclosed in a solid box was mapped in this study; the sequence enclosed in a dashed box is a theoretical region based on the data described in the Discussion. Numbers reflect ovine PrP (ovPrP) sequence positions.

analysis (14), and this binding could be fully blocked in Western blots by a peptide containing residues 94 to 105; these results confirm that somewhere in this region from residues 94 to 105, the N terminus of PrP^{res} in sheep BSE is located. When the results obtained with these two antibodies are considered together with the 1.6-kDa size difference between the aglycosyl fractions of PrP^{res} in sheep BSE and sheep scrapie, it is most likely that the cleavage of PrP^{Sc} in sheep BSE by proteases such as PK occurs mainly in the region from residues 93 to 99 (sheep BSE cleavage site), while in sheep scrapie cleavage occurs about 10 to 17 residues N terminal of this stretch of amino acids (scrapie cleavage site). The low reactivity of P4 with PrP^{res} in sheep BSE and the migration of PrP^{res} in sheep scrapie (Fig. 2) are best explained by the coexistence of a very small population of PrP^{Sc} either in a sheep scrapie conformation or in another folded state which allows proteases to trim PrP^{Sc} up to the sheep scrapie cleavage site (Fig. 7). Alternatively, the P4-reactive bands could be the result of the incomplete action of PK on PrP^{Sc}, yielding a small fraction of larger PrP^{res} molecules.

Since this work was carried out with experimental BSE, it is quite possible that PrP^{res} from sheep with BSE in the field has different biochemical characteristics. Nevertheless, there are a number of factors which make this situation unlikely. We and others used different sources of bovine BSE as an inoculum, different sheep breeds, three different PrP genotypes, and three different routes of infection (1, 17, 20, 22, 35); the results for the brain consistently indicated a smaller molecular size for PrP^{res} bands in sheep BSE than in sheep scrapie. In addition, we showed here that either natural or experimental infection in sheep led to the same molecular PrP^{res} phenotype typical for scrapie, a finding which excludes the possibility that the route of infection has an influence on the molecular behavior of PrP^{Sc}. However, it would be helpful to determine how subsequent passages of sheep scrapie to sheep will behave, since the phenotype of BSE in cattle might be the result of secondary passages (3).

Of the three methods discussed above, diagnosis based on differential antibody binding appears to be the most robust approach for routine screening, since a simple visual inspection leads to an unequivocal result. If the screening initially is performed by a rapid test which is suitable for the detection of both scrapie and BSE in sheep and yields a positive result, the screening should be followed directly by this proposed double-antibody test. So far, this type of analysis has been carried out with different detergents and buffer types with pHs between 7 and 8, indicating that it is quite robust.

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REFERENCES

1. **Baron, T. G. M., J. Y. Madec, D. Calavas, Y. Richard, and F. Barillet.** 2000. Comparison of French natural scrapie isolates with bovine spongiform encephalopathy and experimental scrapie infected sheep. Neurosci. Lett. **284:** 175–178.
2. **Baron, T. G. M., J.-Y. Madec, and D. Calavas.** 1999. Similar signature of the prion protein in natural sheep scrapie and bovine spongiform encephalopathy-linked diseases. J. Clin. Microbiol. **37:**3701–3704.
3. **Baylis, M., F. Houston, R. K. Rowland, A. R. McLean, N. Hunter, and M. B. Gravenor.** 2003. BSE—a wolf in sheep's clothing? Trends Microbiol. **10:**563–570.
4. **Belt, P. B. G. M., I. H. Muileman, B. E. C. Schreuder, J. Bos-de-Ruijter, A. L. J. Gielkens, and M. A. Smits.** 1995. Identifcation of five allelic variants of the sheep PrP gene and their association with natural scrapie. J. Gen. Virol. **76:**509–517.
5. **Bessen, R. A., and R. F. Marsh.** 1994. Distinct PrP properties suggest the molecular basis of strain variation in transmissible mink encephalopathy. J. Virol. **68:**7859–7868.
6. **Bossers, A., R. de Vries, and M. A. Smits.** 2000. Susceptibility of sheep for scrapie as assessed by in vitro conversion of nine naturally occurring variants of PrP. J. Virol. **74:**1407–1414.
7. **Bossers, A., B. E. C. Schreuder, I. H. Muileman, P. B. G. M. Belt, and M. Smits.** 1996. PrP genotype contributes to determining survival times of sheep with natural scrapie. J. Gen. Virol. **77:**2669–2673.
8. **Bruce, M. E., A. Boyle, S. Cousens, I. McConnell, J. Foster, W. Goldmann, and H. Fraser.** 2002. Strain characterisation of natural sheep scrapie and comparison with BSE. J. Gen. Virol. **83:**695–704.
9. **Bruce, M. E., R. G. Will, J. W. Ironside, I. McConnell, D. Drummond, A. Suttie, L. McCardle, A. Chree, J. Hope, C. Birkett, S. Cousens, H. Frase, and C. J. Bostock.** 1997. Transmission to mice indicates that "new variant" CJD is caused by the BSE agent. Nature **389:**498–501.
10. **Bueler, H., M. Fischer, Y. Lang, H. Bluethmann, H. P. Lipp, S. J. DeArmond, S. B. Prusiner, M. Aguet, and C. Weissmann.** 1992. Normal development and behaviour of mice lacking the neuronal cell-surface PrP protein. Nature **356:**577–582.
11. **Burnette, W. N.** 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. **112:**195–203.
12. **Collinge, J., K. C. Sidle, J. Meads, J. Ironside, and A. F. Hill.** 1996. Molecular analysis of prion strain variation and the aetiology of 'new variant' CJD. Nature **383:**685–690.
13. **Foster, J. D., D. W. Parnham, N. Hunter, and M. Bruce.** 2001. Distribution of the prion protein in sheep terminally affected with BSE following experimental oral transmission. J. Gen. Virol. **82:**2319–2326.
14. **Garssen, J. G., L. J. M. van Keulen, C. F. Farquhar, M. A. Smits, J. G. Jacobs, A. Bossers, R. H. Meloen, and J. P. M. Langeveld.** 2000. Applicability of three anti-PrP peptide sera including staining of tonsils and brainstem of sheep with scrapie. Microsc. Res. Techniques **50:**32–39.
15. **Geysen, H. M., R. H. Meloen, and S. J. Barteling.** 1984. Use of peptide synthesis to probe viral antigens for epitopes to a resolution of a single amino acid. Proc. Natl. Acad. Sci. USA **81:**3998–4002.
16. **Harmeyer, S., E. Pfaff, and M. H. Groschup.** 1998. Synthetic peptide vaccines yield monoclonal antibodies to cellular and pathological prion proteins of ruminants. J. Gen. Virol. **79:**937–945.
17. **Hill, A. F., K. C. L. Sidle, S. Joiner, P. Keyes, T. C. Martin, M. Dawson, and J. Collinge.** 1998. Molecular screening of sheep for bovine spongiform encephalopathy. Neurosci. Lett. **255:**159–162.

18. **Hill, A. F., M. Desbruslais, S. Joiner, K. C. L. Sidle, I. Gowland, and J. Collinge.** 1997. The same prion strain causes vCJD and BSE. Nature **389:** 448–450.
19. **Hope, J., G. Multhaup, L. J. Reekie, R. H. Kimberlin, and K. Beyreuther.** 1988. Molecular pathology of scrapie-associated fibril protein (PrP) in mouse brain affected by the ME7 strain of scrapie. Eur. J. Biochem. **172:**271–217.
20. **Hope, J., S. C. E. R. Wood, C. R. Birkett, A. Chong, M. E. Bruce, D. Cairns, W. Goldmann, N. Hunter, and C. J. Bostock.** 1999. Molecular analysis of ovine prion protein identifies similarities between BSE and an experimental isolate of natural scrapie, CH1641. J. Gen. Virol. **80:**1–4.
21. **Hunter, N.** 1997. PrP genetics in sheep and the implications for scrapie and BSE. Trends Microbiol. **5:**331–334.
22. **Hunter, N., J. Foster, A. Chong, S. McCutcheon, D. Parnham, S. Eaton, C. MacKenzie, and F. Houston.** 2002. Transmission of prion diseases by blood transfusion. J. Gen. Virol. **83:**2897–2905.
23. **Jeffrey, M., S. Martin, L. González, S. J. Ryder, S. J. Bellworthy, and R. Jackman.** 2001. Differential diagnosis of infections with the bovine spongiform encephalopathy (BSE) and scrapie agents in sheep. J. Comp. Pathol. **125:**271–284.
24. **Jeffrey, M., S. Ryder, S. Martin, S. A. C. Hawkins, L. Terry, C. Berthelin-Baker, and S. J. Bellworthy.** 2001. Oral inoculation of sheep with the agent of bovine spongiform encephalopathy (BSE). 1. Onset and distribution of disease-specific PrP accumulation in brain and viscera. J. Comp. Pathol. **124:**280–289.
25. **Korth, C., B. Stierli, P. Streit, M. Moser, O. Schaller, R. Fischer, W. Schulz-Schaeffer, H. Kretzschmar, A. Raeber, U. Braun, F. Ehrensperger, S. Hornemann, R.Glockshuber, R. Riek, M. Billeter, K. Wuthrich, and B. Oesch.** 1997. Prion (PrPSc)-specific epitope defined by a monoclonal antibody. Nature **390:**74–77.
26. **Lasmézas, C. I., J.-P. Deslys, R. Demalmay, K. T. Adjou, F. Lamoury, D. Dormont, O. Robain, J. Ironside, and J.-J. Hauw.** 1996. BSE transmission to macaques. Nature **381:**743–744.
27. **Oesch, B., D. Westaway, M. Wälchli, M. P. McKinley, S. B. H. Kent, R. Aebersold, R. A. Barry, P. Tempst, D. B. Teplow, L. E. Hood, S. B. Prusiner, and C. Weissmann.** 1985. A cellular gene encodes scrapie PrP27–30 protein. Cell **40:**735–746.
28. **Parchi, P., W. Zou, W. Wang, P. Brown, S. Capellari, B. Ghetti, N. Kopp, W. J. Schulz-Schaeffer, H. A. Kretzschmar, M. W. Head, J. W. Ironside, P. Gambetti, and S. G. Chen.** 2000. Genetic influence on the structural variations of the abnormal prion protein. Proc. Natl. Acad. Sci. USA **71:**10168–10172.
29. **Prusiner, S. B.** 1982. Novel proteinaceous infectious particles cause scrapie. Science **216:**136–144.
30. **Prusiner, S. B., D. F. Groth, D. C. Bolton, S. B. Kent, and L. E. Hood.** 1984. Purification and structural studies of a major scrapie prion protein. Cell **38:**8322–8327.
31. **Race, R. E., A. Raines, T. G. M. Baron, M. W. Miller, A. Jenny, and E. S. Williams.** 2002. Comparison of abnormal prion protein glycoform patterns from transmissible encephalopathy agent-infected deer, elk, sheep, and cattle. J. Virol. **76:**12365–12368.
32. **Ronaghi, M., M. Uhlén, and P. Nyrén.** 1998. A sequencing method based on real-time pyrophosphate. Science **281:**363–365.
33. **Schaller, O., R. Fatzer, M. Stack, J. Clark, W. Cooley, K. Biffiger, S. Egli, M. Doherr, M. Vandevelde, D. Heim, B. Oesch, and M. Moser.** 1999. Validation of a Western immunoblotting procedure for bovine PrP^{Sc} detection and its use as a rapid surveillance method for the diagnosis of bovine spongiform encephalopthy (BSE). Acta Neuropathol. **98:**437–443.
34. **Schreuder, B. E. C., L. J. M. van Keulen, M. E. W. Vromans, J. P. M. Langeveld, and M. A. Smits.** 1998. Tonsillar biopsy and PrP^{Sc} detection in the preclinical diagnosis of scrapie. Vet. Rec. **142:**564–568.
35. **Stack, M. J., M. J. Chaplin, and J. Clark.** 2002. Diferentiation of prion protein glycoforms from naturally occurring sheep scrapie, sheep-passaged scrapie strains (CH6141 and SSBP1), bovine spongiform encephalopathy (BSE) and Romney and Cheviot breed sheep experimentally inoculated with BSE using two monoclonal antibodies. Acta Neuropathol. **104:**279–286.
36. **Sweeney, T., T. Kuczius, M. McElroy, M. Gomez Parad, and M. H. Groschup.** 2000. Molecular aspects of Irish sheep scrapie cases. J. Gen. Virol. **81:**1621–1627.
37. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
38. **vanKeulen, L. J. M., B. E. C. Schreuder, R. H. Meloen, G. Mooij-Harkes, M. E. W. Vromans, and J. P. M. Langeveld.** 1996. Immunohistochemical detection of prion protein in lymphoid tissues of sheep with natural scrapie. J. Clin. Microbiol. **34:**1228–1231.
39. **vanKeulen, L. J. M., B. E. C. Schreuder, R. H. Meloen, M. Poelen-vanden-Berg, G. Mooij-Harkes, M. E. Vromans, and J. P. M. Langeveld.** 1995. Immunohistochemical detection and localization of prion protein in brain tissue of sheep with natural scrapie. Vet. Pathol. **32:**299–308.
40. **vanKeulen, L. J. M., B. E. C. Schreuder, M. E. W. Vromans, J. P. M. Langeveld, and M. A. Smits.** 2000. Pathogenesis of natural scrapie in sheep. Arch. Virol. **16**(Suppl.)**:**57–71.
41. **Weber, K., and M. Osborn.** 1969. The reliability of molecular weight determination by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. **244:**4406–4412.
42. **Wells, G. A. H., A. C. Scott, C. T. Johnson, R. F. Gunning, R. D. Hancock, M. Jeffrey, M. Dawson, and R. Bradley.** 1987. A novel progressive spongiform encephalopathy in cattle. Vet. Rec. **121:**419–420.
43. **Will, R. G., J. W. Ironside, M. Zeidler, S. N. Cousens, K. Estibeiro, A. Alperovitch, S. Poser, M. Pocchiari, A. Hofman, and P. G. Smith.** 1996. A new variant of Creutzfeldt-Jakob disease in the UK. Lancet **347:**921–925.