Distinct molecular phenotypes in bovine prion diseases

Anne-Gaëlle Biacabe¹, Jean-Louis Laplanche², Stephen Ryder³ & Thierry Baron^{1*}

¹AFSSA-Lyon, Unité 'Virologie-ATNC', Lyon, France, ²UPRES EA 3621, Faculté des Sciences Pharmaceutiques et Biologiques, Université Paris, Paris, France, and ³Neuropathology Unit, Department of Pathology, Veterinary Laboratories Agency, Weybridge, UK

Bovine spongiform encephalopathy (BSE) in cattle, the most likely cause of variant Creutzfeldt–Jakob disease in humans, is thought to be caused by a unique infectious agent, with stable features, even when transmitted to other species. Here, we show the existence of an atypical molecular phenotype among cattle diagnosed with BSE in France. Following western blot analysis, three cases showed unusual features of the electrophoretic profiles of the protease-resistant prion protein (PrP^{res}) accumulating in the brain. The PrP^{res} patterns were similar in these three atypical cases, showing a higher molecular mass of unglycosylated PrP^{res} and strong labelling by P4 monoclonal antibody compared to 55 typical BSE cases. This finding suggests either some phenotypic modifications of PrP^{res} following infection by the BSE agent or the existence of alternative origins of such diseases in cattle.

EMBO reports (2004) 5, 110–114. doi:10.1038/sj.embor.7400054

INTRODUCTION

Bovine spongiform encephalopathy (BSE) is a transmissible spongiform encephalopathy of cattle, first detected in 1986 in the UK and subsequently in other countries. This is the most likely cause of variant Creutzfeldt–Jakob disease (vCJD) in humans (Bruce *et al*, 1997; Hill *et al*, 1997), but the origin of the disease is unknown so far. It may have arisen from an infectious agent causing another similar disease, such as scrapie in sheep and goats. Characterization of the BSE agent derived from a small number of cases by transmission to mice is, however, consistent with infection of cattle by a single strain of infectious agent (Bruce, 1996), which would have been transmitted to cattle following feeding with contaminated meat and bone meal incorporating infected tissues.

Electrophoretic profiles of the protease-resistant form (PrP^{res}) of the disease-associated prion protein accumulating in the brains of BSE-infected animals and humans have demonstrated specific features (Collinge *et al*, 1996; Stack *et al*, 2002). All have shown a lower molecular mass of the unglycosylated PrP^{res} including in humans with vCJD (Collinge *et al*, 1996) and in experimental infections of mice (Kuczius & Groschup, 1999; Baron & Biacabe, 2001) and sheep (Hill *et al*, 1998; Hope *et al*, 1999; Baron *et al*, 2000; Stack *et al*, 2002), in comparison with most other such diseases in these species. Recent studies in transgenic mice expressing the human prion protein nevertheless showed that BSE prions transmitted from cattle BSE could propagate with either vCJD-like or sporadic CJD-like features (Asante *et al*, 2002). However, in cattle BSE, no large-scale studies of the molecular features of PrP^{res} to assess the full uniformity of BSE manifestations or to seek possible other forms of the disease have been reported.

RESULTS

We report the finding of three cattle-BSE cases in France that showed atypical molecular features, among cases diagnosed with the disease following detection of PrP^{res} by the use of rapid western blot or ELISA tests in animals over 24 months old at a slaughterhouse and in rendering plants since 2000 (Morignat *et al*, 2002). All cases were confirmed as BSE-positive using western blot detection of PrP^{res} extracted from the brain stem of the animals, using proteinase K treatment and ultracentrifugation (Madec *et al*, 2000). However, as indicated by the quantities of brain tissues required to obtain comparable PrP^{res} signals between cattle for comparisons of electrophoretic profiles (Figs 1,2), these three samples showed relatively low levels of PrP^{res} in the brain stem. The main features of the three cases with atypical molecular features are summarized in Table 1.

These three cases showed, using identical PrP^{res} extraction and detection procedures in all cases, a different PrP^{res} electrophoretic profile detected by western blot from other cases of cattle BSE. This atypical profile, similar in the three cases, appeared mainly characterized by a higher molecular mass of the unglycosylated PrP^{res} (Fig 1A). An electrophoretic pattern similarly characterized by a higher molecular mass of unglycosylated PrP^{res} was found in a control sample from cattle that had been intracerebrally infected by a British sheep scrapie brain pool (Fig 1A, lane 1). No significant variations were otherwise detected among typical cattle-BSE cases, including in cases showing low levels of PrP^{res} in

¹AFSSA-Lyon, Unité 'Virologie-ATNC', 31 Avenue Tony Garnier, 69364 Lyon Cedex 07, France

²UPRES EA 3621, Faculté des Sciences Pharmaceutiques et Biologiques, Université Paris 5, avenue de l'Observatoire, 75270 Paris Cedex 6, France

³Neuropathology Unit, Department of Pathology, Veterinary Laboratories Agency, Woodham Lane, Weybridge, Surrey KT15 3NE, UK

*Corresponding author. E-mail: t.baron@lyon.afssa.fr

Received 25 September 2003; revised 7 November 2003; accepted 19 November 2003; published online 19 December 2003

Fig 1 | (A) Western blot detection of PrP^{res} from proteinase K-treated and ultracentrifuged brain homogenates, using RB1 polyclonal antibody (105–120 bovine epitope). Atypical cattle-BSE cases (A1_F and A2_F) (lanes 3 and 5) show, as do scrapie experimentally infected cattle (C_{Scr}) (lane 1), a higher molecular mass of the unglycosylated bands (arrow), compared to typical cattle-BSE cases (T_{UK}, lane 2; T1_F, lane 4). Similar differences are also observed in sheep, between natural scrapie (SH_{NS}) (lane 7) and experimental BSE (SH_{BSE}) (lane 6). Brain equivalent quantities loaded per lane from lanes 1 to 7 are 2.4, 4.8, 38, 2.4, 38, 1.2 and 1.2 mg, respectively. (B) Mean molecular weights $\pm$ standard deviations obtained for the di-, mono- and unglycosylated PrP^{res} bands (A: atypical cattle BSE; T: typical cattle BSE; F: French isolate; UK: British isolate; SH: sheep).

the brain stem as was found in the three atypical cases. Comparison with a biotinylated marker of the molecular masses of the three PrP^{res} glycoforms (Fig 1B) showed a 0.7–1.3 kDa difference of the unglycosylated band in atypical cases compared to three typical cattle-BSE cases, from France (two cases) and the UK (one case) (Table 1).

Previous molecular studies showed that typical cattle BSE was also characterized by very weak PrP^{res} labelling by P4 monoclonal antibody using a modification of a western blot rapid test (Stack *et al*, 2002). This finding was confirmed in this study in a series of 55 French cattle-BSE cases diagnosed with the disease between 2000 and 2003, using western blot detection of PrP^{res} following proteinase K treatment and ultracentrifugation (Madec *et al*, 2000). In contrast, PrP^{res} extracted from the brain in the three atypical cases with a higher molecular mass of the unglycosylated form was clearly labelled by P4 antibody, giving PrP^{res} signals comparable to those obtained following detection with RB1 polyclonal antibody as shown in Fig 2.

Glycoform ratios were also measured following detection of PrP^{res} by RB1 polyclonal antibody. It can be seen that, while typical cattle-BSE controls showed the previously described prominent diglycosylated form (Collinge *et al*, 1996; Baron *et al*, 1999b; Stack *et al*, 2002), this was less marked in the three atypical cases, as shown in Fig 3.

Since changes in the sequence of the PrP protein might cause differences in the PrP^{res} electrophoretic patterns, the full sequence of the *prn-p* gene open reading frame was sequenced in two

atypical cases. The results showed a sequence identical to that previously published for the cattle *prn-p* gene (Goldmann *et al*, 1991). Sequencing of five BSE cases among the series of 55 samples that did not react with the P4 monoclonal antibody was also characterized by the same sequence, including the presence of six repeats of the octapeptide region, as found in the three atypical cases.

DISCUSSION

Our results demonstrate the finding of a distinct molecular phenotype of prion diseases in cattle among routinely diagnosed BSE cases following active surveillance of the disease using rapid tests for the detection of PrP^{res} in cattle at a slaughterhouse or in rendering plants. The three cases described here had not been reported as having clinical signs suggestive of BSE during their life and were found in old cattle. This atypical molecular phenotype is mainly characterized by a higher molecular mass of the unglycosylated PrP^{res} and PrP^{res} labelling by P4 monoclonal antibody. This is an unexpected finding since it is believed that this cattle disease is caused by a single strain of infectious agent, which has shown very stable and uniform features, including following its transmission to other species (Bruce, 1996; Collinge *et al*, 1996; Bruce *et al*, 1997; Hill *et al*, 1997; Stack *et al*, 2002). Several hypotheses can be considered to explain this finding.

This may be a manifestation of the BSE agent with different molecular features in cattle, as recently described following transmission in transgenic mice expressing the human prion

protein (Asante *et al*, 2002). Mechanisms involved in such observations remain to be established, but it should be emphasized that such PrP^{res} changes were only found following transmission of cattle BSE, not of human vCJD, to these human transgenic mice.

Genetic differences in the prion genes between these atypical cattle and the general cattle population might be expected to give

rise to variants in electrophoretic profiles of PrP^{res}, as the sequence of the human *PRNP* gene is known to influence the molecular features of PrP^{res} in some cases of human CJD (Cardone *et al*, 1999). Sequencing of the entire open reading frame of the prion genes of two of the atypical cases that showed the known sequence for cattle excluded this hypothesis (Goldmann *et al*, 1991). Importantly, with regard to the single polymorphism described in the bovine *prnp* gene, which can contain five or six repeats of the octapeptide region, no differences were observed between the atypical and typical BSE cases, which could otherwise be distinguished by labelling with P4 monoclonal antibody that recognizes an epitope very close to this region of the protein.

In human CJD, it has also been shown that two distinct PrP^{res} types could be interconverted *in vitro* by altering their metal ion occupancy (Wadsworth *et al*, 1999). Treatment with metal ion chelator EDTA, in the range of concentrations that was shown to modify the PrP^{res} profiles in human CJD, did not modify the electrophoretic patterns of cattle-BSE cases. The differences between atypical cases and typical cattle BSE were maintained, with regard to both molecular mass of unglycosylated PrP^{res} and P4 labelling of PrP^{res}.

Cattle may also have been infected by another source of infectious agent, such as scrapie from sheep and goats. Interestingly, experimental infection of cattle with a British natural sheep scrapie source indeed led to similar differences in the PrP^{res} electrophoretic profiles compared to typical cattle BSE.

Finally, it has been speculated that a spontaneous rare sporadic form of these diseases could exist in cattle as in humans, and might have been the origin of the BSE epidemic. As different PrP^{res} profiles were found between sporadic and variant CJD in humans, this hypothesis might also explain our finding (Collinge *et al*, 1996; Hill *et al*, 2003).

SPECULATION

Further studies are now required to determine the frequency of such novel molecular phenotypes in cattle and the biological features of the involved infecting strain. These may be carried out by means of mouse transmission studies in a panel of wild-type mice with different *prn-p* genotypes (Bruce, 1996), as well as in bovine transgenic mice (Scott *et al*, 1999). However, in a first hypothesis, our results would reinforce the possibility that BSE might have different manifestations, and in this case might be hardly recognized when transmitted to other species as previously suggested (Asante *et al*, 2002; Baron, 2002). Alternatively, this may argue that different forms of the disease may affect cattle,

Fig 2 | Western blot detection of PrP^{res} from proteinase K-treated and ultracentrifuged brain homogenates, using RB1 polyclonal antibody (105–120 bovine epitope) (A) or P4 monoclonal antibody (against the ovine PrP sequence that corresponds to the 97–112 amino acids in cattle) (B). Typical cattle-BSE cases from France (T1_F, lane 2; T2_F, lane 3) or the UK (T_{UK}, lane 1) and atypical cattle-BSE cases (A1_F, A2_F, A3_F; lanes 4, 5, 6, respectively); biotinylated marker (39.8, 29.0, 20.1 and 14.3 kDa bands) (lane 4). Note the position of the lower bands of PrP^{res} detected with RB1 polyclonal antibody in atypical or typical BSE cases compared to the 20.1 kDa band of the biotinylated marker. Brain equivalent quantities loaded per lane are respectively 5.6, 22.4 and 2.8 mg from lanes 1–3 and 37, 56 and 56 mg from lanes 4–7.

Table 1 | Main features of cattle BSE cases characterized by western blot studies

Case	Birth date	Age at death (years)	Geographic origin	Breed	Sex	Origin of samples	Test used for initial diagnosis
A1 _F	15/02/1992	10	Loire Atlantique	Cross-breed	F	Slaughterhouse	Platelia (Biorad)
A2 _F	01/04/1987	15	Côtes d'Armor	Prim'Holstein	F	Rendering plant	Prionics-Check (Prionics AG)
A3 _F	01/05/1993	8	Loire Atlantique	Charolais	F	Rendering plant	Prionics-Check (Prionics AG)
T1 _F	21/04/1993	9	Calvados	Normande	F	Slaughterhouse	Platelia (Biorad)
T2 _F	26/02/1995	7	Ille-et-Vilaine	Prim'Holstein	F	Clinically suspect animal	Prionics-Check (Prionics AG)
T _{UK}	28/08/1994	4	Pontesbury, Shropshire	Cross-breed	F	Clinically suspect animal	Histopathology

Fig 3 | Ratios of di- and monoglycosylated PrP^{res} detected by western blot with RB1 polyclonal antibody in atypical (A1_F, A2_F and A3_F) and typical (T1_F, T2_F and T_{UK}) cattle-BSE cases.

possibly meaning that some cases of such diseases could be detected beyond any possibility of contamination by infected meat-and-bone meal. While contamination and recycling of a scrapie agent in cattle has been a major hypothesis of the origin of BSE, infection of cattle by scrapie agents may have occurred. This may have happened through contamination of feed as possibly occurred at the origin of the BSE epizootic, but direct infection could also be considered since scrapie can be transmitted between sheep and goats by contact and/or through environmental contamination.

METHODS

Animals. Frozen samples from the brain stem of French cattle were obtained following routine testing for BSE diagnosis in cattle over 24 months old at a slaughterhouse and in rendering plants (Morignat *et al*, 2002). Rapid diagnosis tests were used for the detection of PrP^{res} in these samples in routine diagnosis laboratories, using either an ELISA test (Platelia—Biorad) or a western blot test (Prionics-Check—Prionics AG). The presence of PrP^{res} was further evaluated by western blot following proteinase K treatment and ultracentrifugation, as previously described (Madec *et al*, 1998, 2000). Fixed brain materials were not available for histopathological studies.

Brain stem samples from British cattle used as controls were from a natural clinical case of BSE and from a scrapie-infected cow that had been intracerebrally inoculated by a British sheep scrapie source, sampled at the end-terminal stage of the disease.

Extraction of PrP^{res}. Dissociation of 0.35 g fragments of frozen brain tissues was performed in 1.4 ml of 5% glucose in distilled water, in grinding tubes (Biorad), and complete homogenization was obtained by forcing the brain suspension through a 0.4 mm diameter needle. A 600 µl volume was completed to 1.2 ml in 5% glucose, before incubation with proteinase K (10 µg/100 mg brain tissue) (Roche) for 1 h at 37 °C. *N*-lauroyl sarcosyl (30%) (600 µl) (Sigma) was added. Proteinase K digestion thus involved the same

quantities of brain tissues and proteinase K in each sample. After incubation at 20 °C for 15 min, samples were then centrifuged at 200,000g for 2 h on a 10% sucrose cushion, in a Beckman TL100 ultracentrifuge. Pellets were resuspended and heated for 5 min at 100 °C in 30 or 50 µl denaturing buffer (4% SDS, 2% β-mercaptoethanol, 192 mM glycine, 25 mM Tris, 5% sucrose).

Western blot analysis. Samples were run in 15% SDS–PAGE and electroblotted to nitrocellulose membranes in transfer buffer (25 mM Tris, 192 mM glycine, 10% isopropanol) at 400 mA constant during 1 h. The membranes were blocked for 1 h with 5% non-fat dried milk in PBS–Tween 20 (0.1%) (PBST). After two washes in PBST, membranes were incubated (1 h at 20 °C) with RB1 rabbit antiserum (1/2,500 in PBST), raised against synthetic bovine 106–121 (THGQWNKPSKPKTNMK) PrP peptide (Baron *et al*, 1999a), or P4 monoclonal antibody (1/5,000 in PBST), raised against synthetic ovine 89–104 (GGGGWGQGSGHSQWNK) PrP peptide (r-biopharm, Germany) (Harmeyer *et al*, 1998). The corresponding region of the cattle protein recognized by P4 antibody is the 97–112 sequence (GGGGWGQGTHGQWNK). After three washes in PBST, the membranes were incubated (30 min at 20 °C) with peroxidase-labelled conjugates against rabbit or mouse immunoglobulins (1/2,500 in PBST) (Clinisciences). After three washes in PBST, bound antibodies were then detected by Supersignal (Pierce) chemiluminescent substrates, either on films after exposure of the membranes on Biomax MR Kodak films (Sigma) or using pictures obtained with the Fluor-S Multi-imager (Biorad) analysis system. For quantitative studies of the glycoform ratios, chemiluminescent signals corresponding to the three glycoforms of the protein were quantified using the Fluor-S-Multi-imager software. Glycoform ratios were expressed as mean percentages (± standard errors) of the total signal for the three glycoforms (high (H), low (L) and unglycosylated (U) forms), from at least three different runs of the samples. The molecular masses of PrP^{res} glycoforms were precisely evaluated by comparison of the positions of each of the PrP^{res} bands with a biotinylated

marker (B2787, Sigma) using Quantity One (Biorad) software, from six different runs of the samples. Quantities of brain tissues from which PrP^{res} was loaded in each lane are indicated in the figure legends (in milligram brain equivalent).

ACKNOWLEDGEMENTS

We acknowledge the excellent assistance of Katell Peoc'h (UPRES EA 321) in genetic analysis and of Dominique Canal and Jrmy Verchre (AFSSA-Lyon) in western blot analysis.

REFERENCES

- Asante EA, Linehan JM, Desbrulais M, Joiner S, Gowland I, Wood AL, Welch J, Hill AF, Wadsworth JDF, Collinge J (2002) BSE prions propagate as either variant CJD-like or sporadic CJD-like prion strains in transgenic mice expressing human prion protein. *EMBO J* **21**: 6358–6366
- Baron T (2002) Identification of inter-species transmission of prion strains. *J Neuropathol Exp Neurol* **61**: 377–383
- Baron TGM, Biacabe A-G (2001) Molecular analysis of the abnormal prion protein during coinfection of mice by the bovine spongiform encephalopathy agent. *J Virol* **75**: 107–114
- Baron TGM, Betemps D, Groschup MH, Madec JY (1999a) Immunological characterisation of the sheep prion protein expressed as fusion proteins in *Escherichia coli*. *FEMS Immunol Med Microbiol* **25**: 379–384
- Baron TGM, Madec J-Y, Calavas D (1999b) Similar signature of the prion protein in natural sheep scrapie and bovine spongiform encephalopathy-linked diseases. *J Clin Microbiol* **37**: 3701–3704
- Baron TGM, Madec JY, Calavas D, Richard Y, Barillet F (2000) Comparison of French natural scrapie isolates with bovine spongiform encephalopathy and experimental scrapie infected sheep. *Neurosci Lett* **284**: 175–178
- Bruce M (1996) in *Methods in Molecular Medicine: Prion Diseases*, Baker H, Ridley RM (eds) pp 223–236. Totowa, NJ: Humana Press
- Bruce ME et al (1997) Transmissions to mice indicate that 'new variant' CJD is caused by the BSE agent. *Nature* **389**: 498–501
- Cardone F, Liu QG, Petraroli R, Ladogana A, D'Alessandro M, Arpino C, Di Bari M, Macchi G, Pocchiari M (1999) Prion protein glycotype analysis in familial and sporadic Creutzfeldt-Jakob disease. *Brain Res Bull* **49**: 429–433
- Collinge J, Sidle KCL, Meads J, Ironside J, Hill AF (1996) Molecular analysis of prion strain variation and the aetiology of 'new variant' CJD. *Nature* **383**: 685–690
- Goldmann W, Hunter N, Martin T, Dawson M, Hope J (1991) Different forms of the bovine PrP gene have five or six copies of a short, G–C rich element within the protein coding exon. *J Gen Virol* **72**: 201–204
- Harmeyer S, Pfaff E, Groschup MH (1998) Synthetic peptide vaccines yield monoclonal antibodies to cellular and pathological prion proteins of ruminants. *J Gen Virol* **79**: 937–945
- Hill AF, Desbrulais M, Joiner S, Sidle KC, Gowland I, Collinge J, Doey LJ, Lantos P (1997) The same prion strain causes vCJD and BSE. *Nature* **389**: 448–450
- Hill AF, Sidle KCL, Joiner S, Keyes P, Martin TC, Dawson M, Collinge J (1998) Molecular screening of sheep for BSE. *Neurosci Lett* **255**: 159–162
- Hill AF, Joiner S, Wadsworth JDF, Sidle KCL, Bell JE, Budka H, Ironside JW, Collinge J (2003) Molecular classification of sporadic Creutzfeldt-Jakob disease. *Brain* **126**: 1333–1346
- Hope J, Wood SCER, Birkett CR, Chong A, Bruce ME, Cairns D, Goldmann W, Hunter N, Bostock CJ (1999) Molecular analysis of ovine prion protein identifies similarities between BSE and an experimental isolate of natural scrapie, CH 1641. *J Gen Virol* **80**: 1–4
- Kuczius T, Groschup MH (1999) Differences in proteinase K resistance and neuronal deposition of abnormal prion proteins characterise bovine spongiform encephalopathy (BSE) and scrapie strains. *Mol Med* **5**: 406–418
- Madec JY, Groschup MH, Buschmann A, Belli P, Calavas D, Baron T (1998) Sensitivity of the western blot detection of prion protein PrP^{res} in natural sheep scrapie. *J Virol Methods* **75**: 169–177
- Madec JY, Belli P, Calavas D, Baron T (2000) Efficiency of western blotting for the specific immunodetection of proteinase K-resistant prion protein in BSE diagnosis in France. *Vet Rec* **146**: 74–76
- Morignat E et al (2002) Targeted surveillance to assess the prevalence of BSE in high-risk populations in Western France and the associated risk factors. *Vet Rec* **151**: 73–77
- Scott MR, Will R, Ironside J, Nguyen H-O, Tremblay P, DeArmond SJ, Prusiner SB (1999) Compelling transgenic evidence for transmission of bovine spongiform encephalopathy prions to human. *Proc Natl Acad Sci USA* **96**: 15137–15142
- Stack M, Chaplin M, Clark J (2002) Differentiation of prion protein glycoforms from naturally occurring sheep scrapie, sheep-passaged scrapie strains (CH1641 and SSBP1), bovine spongiform encephalopathy (BSE) cases and Romney and Cheviot breed sheep experimentally inoculated with BSE using two monoclonal antibodies. *Acta Neuropathol* **104**: 279–286
- Wadsworth JDF, Hill AF, Joiner S, Jackson GS, Clarke AR, Collinge J (1999) Strain-specific prion protein conformation determined by metal ions. *Nat Cell Biol* **1**: 55–59