Distinct molecular phenotypes in bovine prion diseases

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INTRODUCTION

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 (PrPres) accumulating in the brain. The PrPres patterns were similar in these three atypical cases, showing a higher molecular mass of unglycosylated PrPres and strong labelling by P4 monoclonal antibody compared to 55 typical BSE cases. This finding suggests either some phenotypic modifications of PrPres following infection by the BSE agent or the existence of alternative origins of such diseases in cattle.


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 PrPres 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 PrPres 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 PrPres signals between cattle for comparisons of electrophoretic profiles (Figs 1,2), these three samples showed relatively low levels of PrPres 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 PrPres extraction and detection procedures in all cases, a different PrPres 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 PrPres (Fig 1A). An electrophoretic pattern similarly characterized by a higher molecular mass of unglycosylated PrPres 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 PrPres in
the brain stem as was found in the three atypical cases. Comparison with a biotinylated marker of the molecular masses of the three PrPres 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 PrPres 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 PrPres following proteinase K treatment and ultracentrifugation (Madec et al., 2000). In contrast, PrPres 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 PrPres signals comparable to those obtained following detection with RB1 polyclonal antibody as shown in Fig 2.

Glycoform ratios were also measured following detection of PrPres 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 PrPres electrophoretic patterns, the full sequence of the \emph{prnp} gene open reading frame was sequenced in two atypical cases. The results showed a sequence identical to that previously published for the cattle \emph{prnp} 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 PrPres 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 PrPres and PrPres 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 PrPres 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 PrPres, as the sequence of the human PRNP gene is known to influence the molecular features of PrPres 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 PrPres 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 PrPres 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 PrPres and P4 labelling of PrPres.

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 PrPres 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,

<table>
<thead>
<tr>
<th>Case</th>
<th>Birth date</th>
<th>Age at death (years)</th>
<th>Geographic origin</th>
<th>Breed</th>
<th>Sex</th>
<th>Origin of samples</th>
<th>Test used for initial diagnosis</th>
</tr>
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<tr>
<td>A1F</td>
<td>15/02/1992</td>
<td>10</td>
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<td>F</td>
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<td>Platelia (Biorad)</td>
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<td>15</td>
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<td>Prim’Holstein</td>
<td>F</td>
<td>Rendering plant</td>
<td>Prionics-Check (Prionics AG)</td>
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<tr>
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<td>01/05/1993</td>
<td>8</td>
<td>Loire Atlantique</td>
<td>Charolais</td>
<td>F</td>
<td>Rendering plant</td>
<td>Prionics-Check (Prionics AG)</td>
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<td>9</td>
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<tr>
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<td>28/08/1994</td>
<td>4</td>
<td>Pontesbury, Shropshire</td>
<td>Cross-breed</td>
<td>F</td>
<td>Clinically suspect animal</td>
<td>Histopathology</td>
</tr>
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</table>
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 environ-
mental 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\textsuperscript{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
PrPres 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
obtained 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\textsuperscript{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 \mu l volume was completed to 1.2 ml in 5%
glucose, before incubation with proteinase K (10 \mu g/100 mg brain
tissue) (Roche) for 1 h at 37 °C. N-lauroyl sarcosyl (30%) (600 \mu l)
(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
200,000 g 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 \mu l denaturing buffer (4% SDS, 2% 
\beta-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 (GGGGWGQGGSHSQWNK) 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 (GGGWGQGGTHGQWNK).

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) (Clini-
siences). After three washes in PBST, bound antibodies were
then detected by Supersignal (Pierce) chemiluminescent sub-
strates, 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\textsuperscript{res} glycoforms were precisely evaluated by compar-
ison of the positions of each of the PrP\textsuperscript{res} bands with a biotinylated

![Fig 3: Ratios of di- and monoglycosylated PrP\textsuperscript{res} detected by western blot with RB1 polyclonal antibody in atypical (A1\textsubscript{F}, A2\textsubscript{F} and A3\textsubscript{F}) and typical (T1\textsubscript{F},
T2\textsubscript{F} and T\textsubscript{UK}) cattle-BSE cases.](image-url)
marker (B2787, Sigma) using Quantity One (Biorad) software, from six different runs of the samples. Quantities of brain tissues from which PrPres was loaded in each lane are indicated in the figure legends (in milligram brain equivalent).

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REFERENCES