BSE prions propagate as either variant CJD-like or sporadic CJD-like prion strains in transgenic mice expressing human prion protein


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Variant Creutzfeldt–Jakob disease (vCJD) has been recognized to date only in individuals homozygous for methionine at PRNP codon 129. Here we show that transgenic mice expressing human PrP methionine 129, inoculated with either bovine spongiform encephalopathy (BSE) or variant CJD prions, may develop the neuropathological and molecular phenotype of vCJD, consistent with these diseases being caused by the same prion strain. Surprisingly, however, BSE transmission to these transgenic mice, in addition to producing a vCJD-like phenotype, can also result in a distinct molecular phenotype that is indistinguishable from that of sporadic CJD with PrPSc type 2. These data suggest that more than one BSE-derived prion strain might infect humans; it is therefore possible that some patients with a phenotype consistent with sporadic CJD may have a disease arising from BSE exposure.

Keywords: BSE/Creutzfeldt–Jakob disease/prion/transgenic

Introduction

Prion diseases, such as Creutzfeldt–Jakob disease (CJD) in humans, and scrapie and bovine spongiform encephalopathy (BSE) in animals, are transmissible neurodegenerative diseases associated with accumulation of a disease-associated isoform of host-encoded cellular prion protein (PrPc), designated PrPSc. PrPSc is thought to comprise an aggregated form of a conformational isomer of PrPc. According to the protein-only hypothesis, infectious prions are composed principally, if not entirely, of an abnormal isoform of PrP. Distinctive isolates or strains of prions can be propagated in the same type of host and may be encoded by differences in PrPSc conformation (Bessen and Marsh, 1992, 1994; Collinge et al., 1996b; Telling et al., 1996) and glycosylation (Collinge et al., 1996b). Variant CJD (vCJD), recognized in 1996, is thought to be caused by exposure to BSE-like prions (Collinge et al., 1996b; Lasmézas et al., 1996; Bruce et al., 1997; Hill et al., 1997). We have previously described four human PrPSc types in brain tissue from patients with CJD: types 1–3 are seen in classical (sporadic or iatrogenic) CJD, while type 4 is seen in vCJD (Collinge et al., 1996b).

A common polymorphism at codon 129 of the human PrP gene (PRNP), where either methionine (M) or valine (V) can be encoded, is a key determinant of susceptibility to sporadic and acquired prion diseases, and may affect age at onset in inherited prion disease (Baker et al., 1991; Collinge et al., 1991; Palmer et al., 1991). To date, all patients recognized with vCJD have been of the PRNP 129MM genotype (Collinge et al., 1996a; Zeidler et al., 1997; our unpublished data). PrP polymorphisms are known to affect prion strain propagation in mice and sheep (Bruce, 1993). Similarly, codon 129 genotype may play a role in human prion strain propagation, since certain PrPSc types are closely associated with codon 129 genotypes. To date, we have found types 1 and 4 PrPSc only in individuals of the PRNP 129MM genotype and type 3 PrPSc only in genotypes MV or VV, while type 2 PrPSc is seen in association with all three genotypes (Collinge et al., 1996b; Wadsworth et al., 1999; our unpublished data). We have previously reported that Tg(HuPrP129V+/+ Prnp0/0), 152 mice, which express only human PrP V129 (129VV Tg152 mice), are highly susceptible to infection with human prions from patients with sporadic and iatrogenic forms of CJD, regardless of patient genotype at polymorphic codon 129 (Collinge et al., 1995; Hill et al., 1997). However, these mice are much less susceptible to prions from patients with vCJD. Indeed, the transmission properties of vCJD closely resembled those of BSE, and these experiments form part of the extensive data arguing that vCJD is caused by a BSE-like prion strain (Collinge et al., 1996b; Bruce et al., 1997; Hill et al., 1997). These mice lacked a species or transmission barrier to classical CJD prions and were also used to model the transmission barrier between cattle and humans (Collinge et al., 1995; Hill et al., 1997). These data were relatively reassuring, in that transmission of BSE to transgenic mice expressing only human PrP was inefficient, with <40% of intracerebrally inoculated mice succumbing to prion disease after prolonged incubation periods, consistent with the presence of a substantial transmission barrier. However, an important caveat with respect to public health considerations was that vCJD was occurring in humans of the PRNP 129MM genotype, while these mice expressed human PrP 129V (Collinge et al., 1995; Hill et al., 1997). Although classical CJD from patients with all three PRNP codon 129 genotypes (MM, VV and MV) transmitted efficiently to these mice, it is possible that part of the transmission barrier to vCJD infection of these mice resided in the mismatch at codon 129 between inoculum and host (Hill et al., 1997). Using the same inocula, we have now extended these studies to mice expressing human PrP M129 to further study both the bovine-to-human species
barrier and the propagation of human and BSE prion strains. Detailed study of the relative transmission barriers to BSE in transgenic mice expressing human PrP M129 and V129 will be published elsewhere. Here we report the unexpected finding that BSE prion inoculation can induce replication of two distinct prion strains in mice expressing human prion protein.

Results

Susceptibility of transgenic mice expressing human PrP M129 to human and bovine prions

We produced transgenic mice homozygous for a human PrP M129 transgene array and murine PrP null (Bueler et al., 1992) alleles (Prnp0/0), designated Tg(HuPrP129M+/+ Prnp0/0)-35 (129MM Tg35), with expression levels of human PrP two times that of pooled normal human brain (data not shown). These mice were challenged with prions from cases of sporadic CJD, vCJD and BSE. 129MM Tg35 mice were highly susceptible to prions from patients with sporadic CJD of the PRNP 129MM genotype, but were less susceptible to classical CJD prions from individuals of the PRNP 129VV genotype (Table I). Transmission of sporadic CJD of the PRNP 129MV genotype was associated with either consistent short-duration characteristics as with MM cases (I024) or long and variable incubation periods (I020). This may reflect stochastic propagation of either 129M or 129V PrPSc in these patients. This was in contrast to Tg(HuPrP129VPrp109)-152 mice, expressing human PrP V129 (129VV Tg152), which, as we have reported previously (using the same inocula), are highly susceptible to classical CJD prions from all three PRNP genotypes (Collinge et al., 1995; Hill et al., 1997). The presence of a transmission barrier can be estimated by measuring the fall in mean incubation period on primary and second passage in the same host. Second passage of prions from sporadic CJD (I1202)-inoculated 129MM Tg35 mice resulted in an incubation period of 249 ± 3 days (4/4 mice), which was not lower than primary passage [229 ± 5 days (8/8 mice)]. It is possible that the small increase in incubation period reflects a lower prion titre in mouse than human brain since affected mice are culled at an early clinical stage. Consistent short incubation periods on primary passage with 100% attack rate and no fall in incubation period on second passage of CJD in these mice, as with our earlier studies with Tg152 mice (Collinge et al., 1995), are consistent with lack of a transmission barrier to classical CJD 129MM prions. However, as with 129VV Tg152 mice (Hill et al., 1997), 129MM Tg35 mice were much more resistant to vCJD 129MM prions, with only 1/14 mice succumbing to clinical prion disease at a prolonged incubation period (690 days) (Tables I and II). Indeed, as judged by development of clinical disease, 129MM Tg35 mice, expressing human PrP 129M, appeared less susceptible to vCJD than 129VV Tg152 mice, expressing human PrP 129V (Hill et al., 1997). Similarly, 129MM Tg35 mice appeared highly resistant to BSE prions, with 6/49 clinically scored transmissions at variable and prolonged incubation periods (338±492 days) (Tables I and II).

Sub-clinical infection in mice expressing human PrP M129

While by clinical criteria these data might be interpreted as consistent with the existence of a substantial species barrier between cattle BSE and transgenic mice expressing 129M human PrP, we investigated all these mice for evidence of sub-clinical infection. We and others have previously demonstrated extensive sub-clinical prion infection in mice inoculated with a strain of hamster prions (Sc237 or 263K) thought to be non-pathogenic to wild-type mice (Hill et al., 2000; Race et al., 2001),

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Code</th>
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<th>Human PrPSc type</th>
<th>Clinical signs</th>
<th>Incubation period (days ± SEM)</th>
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<td>229 ± 5</td>
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<td>T1</td>
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<td>225 ± 7</td>
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<td>&gt;500</td>
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aPositive either by clinical signs, western blotting and/or immunohistochemistry.
bGenotype at corresponding bovine PrP gene codon.
cOne brain not available for either western blotting or immunohistochemistry.

Table I. vCJD, BSE and sporadic CJD transmissions to transgenic mice expressing human PrP 129M

Transgenic modelling of BSE and vCJD
questioning current definitions of transmission barriers, which have been conventionally assessed on the basis of occurrence of clinical disease in inoculated animals. Surprisingly, as assessed by histology, immunohistochemistry and/or the presence of human PrPSc on western blotting of brain tissue, we found that all (13/13) vCJD-inoculated 129MM Tg35 mice, which had died apparently of age-related causes without clinical signs of prion disease at ages typical for un inoculated or mock-inoculated mice, had pathological (Figure 1A) and/or biochemical (Figure 2A) evidence of prion infection. Only a single (1/14) (Table II) 129MM Tg35 mouse challenged with vCJD developed clinical prion disease. Excluding those animals that died soon after inoculation, which were

Table II. Summary of BSE and vCJD transmission to Tg35 and Tg45

| Transgenic line | BSE |   |   |   | vCJD |   |   |   |   |   |   |   |
|----------------|-----|---|---|---|-----|---|---|---|---|---|---|---|---|
|                | Total attack rate | Clinical disease | Sub-clinical infection | Type 2 PrPSc | Type 4 PrPSc | Total attack rate | Clinical disease | Sub-clinical infection | Type 2 PrPSc | Type 4 PrPSc |
| Tg35           | 14/49 | 6/49 | 8/49 | 10/11 | 1/11 | 14/14 | 1/14 | 13/14 | 0/14 | 14/14 |
| Tg45           | 9/12 | 0/12 | 9/12 | 0/9 | 9/9 | 4/4 | 1/4 | 3/4 | 0/4 | 4/4 |

*Three brains not analysed by western blotting (one brain from a clinically infected animal was unavailable for either western blotting or immunohistochemistry; single brains from clinically affected and sub-clinically affected animals were scored positive by immunohistochemistry).

**Fig. 1.** Immunohistochemistry of cerebral cortex and hippocampal regions of transgenic mouse brain showing abnormal PrP immunoreactivity, including PrP-positive florid plaques (enlarged in insets). (A) vCJD-inoculated 129MM Tg35 mouse. (B) BSE-inoculated 129MM Tg35 mouse. (C) vCJD-inoculated 129MM Tg45 mouse. (D) BSE-inoculated 129MM Tg45 mouse. (E–G) Histological analysis showing the thalamus of a BSE-inoculated 129MM Tg35 mouse propagating type 2 human PrPSc with widespread vacuolation (E; H&E), extensive gliosis (F; GFAP), but no specific PrP immunoreactive deposits (G; ICSM 35). Scale bar: (A–D) = 100 μm; (E), (F) and (G) = 50 μm.
Fig. 2. Western blots of proteinase K (PK)-treated brain homogenates from transgenic mice, human cases of variant and sporadic CJD, and lines of wild-type mice. (A) Lane 1, vCJD; lane 2, vCJD-inoculated 129MM Tg35 mouse. (B) Lane 1, vCJD-inoculated 129MM Tg35 mouse; lane 2, BSE-inoculated 129MM Tg35 mouse propagating type 2 PrPSc; lane 3, vCJD. (C) Lanes 1 and 2, BSE-inoculated 129MM Tg35 mouse propagating either type 2 PrPSc (lane 1) or type 4 PrPSc (lane 2). (D) Lane 1, BSE-inoculated 129MM Tg35 mouse propagating type 2 PrPSc; lane 2, human sporadic CJD type 2 PrPSc (PRNP genotype 129MM). (E) Lanes 1–3, human sporadic CJD type 2 PrPSc (PRNP genotype 129MM); lanes 4–6, BSE-inoculated 129MM Tg35 mouse propagating type 2 PrPSc. Samples were PK digested in the absence (lanes 1, 3, 4 and 6) or presence (lanes 2 and 5) of 25 mM EDTA. *Following proteolysis, samples in lanes 3 and 6 were boiled in SDS sample buffer and subsequently adjusted to 25 mM EDTA before electrophoresis. (F) Transmission of vCJD and BSE to 129MM Tg45 mice. Lane 1, vCJD; lane 2, vCJD-inoculated 129MM Tg45 mouse; lane 3, BSE-inoculated 129MM Tg45 mouse. (G) Primary transmission of vCJD and BSE to wild-type inbred mice. Lane 1, BSE-inoculated FVB mouse; lane 2, vCJD-inoculated FVB mouse; lane 3, BSE-inoculated C57BL/6 mouse; lane 4, BSE-inoculated SJL mouse; lane 5, vCJD-inoculated SJL mouse; lane 6, BSE-inoculated RHIS mouse. (H) Secondary transmission of vCJD and BSE in wild-type inbred mice. Lanes 1–4, BSE was passaged twice in C57BL/6 mice and then passaged in different wild-type mice: lane 1, C57BL/6 mouse; lane 2, FVB mouse; lane 3, SJL mouse; lane 4, RHIS mouse; lanes 5 and 6, second passage of SJL-passaged BSE in further SJL mice (lane 5) or FVB mice (lane 6). Western blots were analysed by high-sensitivity ECL using biotinylated anti-PrP monoclonal antibody ICSM 35 (A–D, F–H) or 3F4 (E).

Transgenic modelling of BSE and vCJD

Transgenic mice expressing human PrP M129 develop the neuropathological features and PrPSc type of vCJD following inoculation with BSE or vCJD prions

Inoculation of vCJD prions into 129MM Tg35 mice resulted in clinical disease in only a single mouse, with widespread sub-clinical disease with human PrPSc readily detectable in brain by western blot analysis. In previous transmission studies of vCJD prions to 129Vv Tg152 mice, a novel type 5 PrPSc pattern was obtained, and thought to represent a prion strain switch resulting from mismatch of the codon 129 polymorphism in inoculum and host human PrP (Hill et al., 1997). A prediction of the hypothesis that prion strain type is encoded by PrPSc structural properties, and that the PRNP codon 129 polymorphism plays a key role in human prion strain propagation, is that transmission of vCJD prions (containing human PrPSc type 4) to 129MM Tg35 mice would result in faithful propagation of type 4 PrPSc. This was indeed what we observed: the PrPSc type seen, as judged by PrPSc fragment sizes (Figure 2A, compare lanes 1 and 2), was the type 4 pattern characteristic of vCJD prions in human brain. The glycoform ratio also closely resembled that of type 4 PrPSc in human brain (Figure 3). As we have reported previously, a small difference is seen on glycoform ratios of the same prion strain propagated in mice and human brain, presumably reflecting the superimposition of species-specific glycosylation effects on the prion strain-specific pattern (Hill et al., 1997). Furthermore, the neuropathological features in the vCJD-inoculated 129MM Tg35 mice were quite different from those of 129Vv Tg152 mice propagating type 5 human PrPSc, where no PrP immunoreactive plaques were seen (Hill et al., 1997). Remarkably, the vCJD-inoculated 129MM Tg35 mice not only developed abundant PrP plaques, an uncommon feature of prion disease in mice, but many of these were of the ‘florid’ type (a central plaque core surrounded by a ring of spongiform vacuoles), which are characteristic of vCJD in humans (Will et al., 1996) (Figure 1A) but rarely seen in mice. Florid plaques were first described in Icelandic scrapie and have also been described in mice infected with the 111A scrapie strain (McBride et al., 1988). More recently, florid plaques have been reported in BSE-inoculated primates (LasmeÁzas et al., 1996) and in transgenic mice expressing ovine PrP infected with sheep-passaged BSE prions (Crozet et al., 2001).
BSE prion inoculation of 129MM Tg35 mice also resulted in both clinical disease and sub-clinical infection (Tables I and II). In sharp contrast to BSE transmission to 129VV Tg152 mice, where we were unable to detect PrPSc in brain (Hill et al., 1997), PrPSc was readily detectable in brains of clinically sick 129MM Tg35 mice and in mice not showing clinical signs of prion disease when they died at advanced age (Figures 2C and 3). In one of the eight sub-clinically affected mice, type 4 human PrPSc was seen (Figure 2C, lane 2), indistinguishable from that seen in vCJD-inoculated 129MM Tg35 mice and in human vCJD itself. In this mouse, neuropathological features were identical to those of vCJD-inoculated mice, with abundant florid plaques as in human vCJD (Figure 1B). These data further supported the conclusion that vCJD is caused by a BSE-like prion strain. However, in all other sub-clinically affected BSE-inoculated 129MM Tg35 mice (7/8), an alternate phenotype was observed (Table II). This was also seen in all clinically affected BSE-inoculated 129MM Tg35 mice where brain was available for analysis (5/6) (Table II).

Some Tg(HuPrPM129) mice develop a distinct phenotype following inoculation with BSE prions
In 4/6 clinically affected and 6/8 sub-clinically affected BSE-inoculated 129MM Tg35 mice, a distinctive human PrPSc type was seen with a quite different fragment size of unglycosylated PrPSc following proteinase K digestion and a different ratio of the three glycoforms, monoglycosylated PrPSc being most abundant (in marked contrast to type 4 PrPSc, where diglycosylated PrPSc predominates) (Figure 2B, C and E, compare lanes 1 and 2). Comparison with known human PrPSc types in CJD indicated that this type corresponded, both with respect to fragment sizes and glycoform ratio, to the type 2 PrPSc seen in sporadic and iatrogenic CJD (Figure 2D, compare lanes 1 and 2, and Figure 3). Human PrPSc types can also be distinguished by their metal-binding properties. Both type 1 and type 2 human PrPSc undergo a shift in fragment size following proteinase K treatment if treated with the metal chelator EDTA (Wadsworth et al., 1999). Type 3 (also seen in classical CJD) and type 4 PrPSc do not undergo a metal-dependent shift in proteinase K cleavage site on treatment with EDTA (Wadsworth et al., 1999). Treatment of BSE-inoculated 129MM Tg35 mouse brain homogenates with EDTA (Wadsworth et al., 1999). Treatment of BSE-inoculated 129MM Tg35 mouse brain homogenates with EDTA prior to proteinase K digestion and the characteristic PrPSc type of vCJD is maintained in 129MM Tg35 mice similar to that seen in human vCJD, and the characteristic PrPSc type of vCJD is maintained in all mice, BSE inoculation results in two distinct but highly consistent phenotypes: one indistinguishable from the vCJD transmissions, and associated with the characteristic molecular ‘signature’ of BSE; and a second that resembles transmission of the commonest molecular sub-type of classical CJD.

vCJD and BSE transmission to a further HuPrP129M-expressing transgenic line
We also inoculated a second transgenic line expressing HuPrP129M, generated as described for Tg35, with vCJD and BSE prions. Tg(HuPrP129M<sup>+/-</sup> Pmri<sup>1024</sup>-45) (129MM Tg45) mice were produced similarly to 129MM Tg35 mice, but have a level of expression of human PrP 4-fold higher than a pooled normal human brain standard (data not shown). These mice were also highly susceptible to sporadic CJD, with a 100% attack rate, extremely short and consistent incubation periods (I024: 7/7 mice developed disease with an incubation time of 155 ± 5 days), and no fall in incubation period on second passage, consistent with lack of a transmission barrier to classical CJD prions. Again, as judged by clinical disease, we found that these animals were much less susceptible to vCJD and BSE. However, as seen with BSE- or vCJD-inoculated 129MM Tg35 mice, evidence of sub-clinical prion infection was seen in most clinically unaffected mice (Table II). While only 1/4 vCJD-inoculated 129MM Tg45
mice developed clinical disease (at 580 days), the remaining 3/4 mice had neuropathological and biochemical evidence of prion infection. Again, in close agreement with the results from 129MM Tg35 mice, analysis of brains of vCJD-inoculated 129MM Tg45 mice consistently revealed widespread florid plaque deposition (Figure 1C) and type 4 PrPSc (Figure 2F, lane 2 and Figure 3). Similarly, none of the BSE-inoculated 129MM Tg45 mice developed clinical signs of prion disease for >700 days, but 9/12 had sub-clinical prion infection (Table II). Neuropathological examination of BSE-inoculated 129MM Tg45 mice revealed closely similar pathological findings to that of vCJD-inoculated 129MM Tg45 mice with florid plaques (Figure 1D) and western blot analysis of brain tissue revealed type 4 PrPSc (Figure 2F, lane 3 and Figure 3). To date, the alternate neuropathological pattern associated with type 2 PrPSc has not been detected in BSE-inoculated 129MM Tg45 mice.

vCJD and BSE transmission to various inbred lines of non-transgenic mice

BSE prions transmit readily to wild-type mice but with prolonged and variable incubation periods. We have previously reported the PrPSc type of both FVB and C57BL/6 mice when inoculated with BSE (Collinge et al., 1996b; Hill et al., 1997). As with other species naturally or experimentally infected with BSE that we have reported, a BSE-like pattern is produced with a characteristic PrPSc fragment size and glycoform ratio. However, these transmissions involve PrP from another mammalian species of different molecular mass, such that the proteins are not directly comparable, as with transmissions of human prion disease to transgenic mice expressing only human PrP. This mouse PrPSc pattern is, therefore, referred to as 'diglycosylated dominant'.

In independent experiments to determine the range of incubation periods of BSE in many inbred mouse lines, as part of studies to map prion incubation time genes (Lloyd et al., 2001), we have identified two inbred lines of mice in which BSE transmission is associated with the production of a distinctive PrPSc type, with PrPSc glycoform ratios closely similar to that of human sporadic CJD and referred to here as a 'monoglycosylated dominant' PrPSc pattern. Interestingly, these lines are also associated with unusually short incubation periods for BSE (Table III). All four inbred mouse lines have the same Prnp coding sequence (Prnp-a; data not shown) and are homozygous for methionine at codon 128, the corresponding murine codon to PRNP codon 129.

Following inoculation with BSE prions, both FVB and C57BL/6 mice show the characteristic diglycosylated dominant PrPSc pattern in the brain (Figures 2G, lanes 1 and 3, and 4A). However, when inoculated with the same BSE inoculum, SJL and RIIIIs mice exhibit a monoglycosylated dominant PrPSc pattern (Figures 2G, lanes 4 and 6, and 4A). This PrPSc type is stable on further passage to both SJL and FVB mice (Figures 2H, lanes 5 and 6, and 4C) and is unaffected by EDTA treatment (data not shown).

The propagation of the monoglycosylated PrPSc glycoform pattern is established by the host in which primary passage is carried out, as both SJL and RIIIIs mice are capable of propagating the diglycosylated dominant PrPSc pattern when challenged with BSE passaged twice in a C57BL/6 mouse (Figures 2H, lanes 3 and 4, and 4C).

vCJD prions behave in the same way as BSE prions in FVB mice, producing a prolonged and variable incubation period and a diglycosylated dominant PrPSc type (Hill et al., 1997) (Figures 2G, lane 2, and 4A; Table III). vCJD transmissions to SJL mice also resemble BSE transmissions to these mice. Inoculation with vCJD gives unusually short incubation times (Table III) and produces a monoglycosylated dominant PrPSc pattern which is closely similar to that produced by BSE transmission (Figures 2G, lane 5, and 4A) and is unaffected by EDTA treatment (data not shown). To our knowledge, the PrPSc pattern seen on BSE or vCJD transmissions to RIIIIs and SJL mice used in our study, or to the RIII strain of mice routinely used for biological strain typing experiments, has not been reported previously (Bruce et al., 1997).

The neuropathological features seen in inbred lines of mice inoculated with the same prion strain vary considerably, the disease patterns being host, as well as prion strain, dependent (Bruce, 1993). The neuropathology observed in SJL and RIIIIs mice inoculated with either BSE or vCJD showed only diffuse staining for PrP without florid or other PrP immunoactive plaques (data not shown).

### Table III. BSE and vCJD transmissions to inbred lines of mice

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<th>FVB/NHsd</th>
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<td>Incubation time (days ± SEM)</td>
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**Discussion**

Prion propagation involves recruitment and conversion of host PrPSc into PrPSc, and the degree of primary structural similarity between inoculated PrPSc and host PrPSc is thought to be a key component of intermammalian transmission barriers (Prusiner et al., 1990). It is clear, however, that prion strain type can also be crucial, as clearly demonstrated by the very distinctive transmission...
properties of sporadic CJD 129MM and low molecular mass (diglycosylated) and low molecular mass (monoglycosylated) glycoforms seen in sporadic CJD, vCJD, BSE and in wild-type mice following challenge with vCJD and BSE. Data points are plotted as mean ± SEM. (A–C) Human cases indicated as circles: sporadic CJD type 1 PrPSc, light grey (n = 12); sporadic CJD type 2 PrPSc, mid-grey (n = 49); sporadic CJD type 3 PrPSc, dark grey (n = 22); vCJD type-4 PrPSc, yellow (n = 16). Cattle BSE, black square (n = 3). (A) Primary transmission of vCJD and BSE to wild-type mice: vCJD-inoculated FVB mice, green diamond (n = 19); vCJD-inoculated SJL mice, green triangle (n = 4); BSE-inoculated FVB mice, red diamond (n = 12); BSE-inoculated SJL mice, red triangle (n = 7); BSE-inoculated RIIB mice, red star (n = 4); BSE-inoculated C57BL/6 mice, inverted red triangle (n = 3). (B) Transmission of SJL-passaged BSE to further wild-type mice: SJL-passaged-BSE-inoculated FVB mice, blue diamond (n = 4); BSE passed twice in SJL mice, blue triangle (n = 3); BSE passed three times in SJL mice, open triangle (n = 3). (C) Transmission of BSE passed twice in C57BL/6 mice to further wild-type mice: C57BL/6-passaged BSE to FVB mice, orange diamond (n = 3); C57BL/6-passaged BSE to SJL mice, orange triangle (n = 4); C57BL/6-passaged BSE to RIIB mice, orange star (n = 3); C57BL/6-passaged BSE to C57BL/6 mice, inverted orange triangle (n = 3).

129MM Tg35 mice further argues for the need to reassess current definitions of ‘species’ or transmission barriers that limit prion transmission between different hosts (Hill et al., 2000). Such barriers have hitherto been quantitated on the basis of either comparative end-point titrations in the two respective hosts, or by measuring the fall in incubation period between primary and subsequent passage as the prion strain adapts to the new host. Both methods rely on measurement of time to onset of a clinical syndrome. Modelling the BSE-to-human barrier in 129MM Tg35 mice would lead to the conclusion, on the basis of induced clinical disease, that a substantial barrier existed. However, it is clear that human PrPSc propagation can be efficiently induced by inoculation with BSE or vCJD prions, suggesting a smaller barrier to infection (but not to clinical disease) than hitherto thought (Collinge et al., 1995) in humans of the PRNP 129MM genotype. Humans infected with BSE prions, but who became asymptomatic carriers, may nevertheless pose a threat of iatrogenic transmission via medical and surgical procedures. Alternatively, it is possible that the lifespan of the laboratory mouse is insufficient to allow expression of clinical disease in most inoculated mice, whereas a higher proportion of infected humans might survive the incubation period to develop clinical signs of disease. Serial passage studies and titration of prions in these mice are in progress to study this further.

These studies further strengthen the evidence that vCJD is caused by a BSE-like prion strain. Also, remarkably, the key neuropathological hallmark of vCJD, the presence of abundant florid PrP plaques, can be recapitulated on BSE or vCJD transmission to these mice. However, the most surprising aspect of the studies was the finding that an alternate pattern of disease can be induced in 129MM Tg35 mice from primary transmission of BSE, with a molecular phenotype indistinguishable from that of a subtype of sporadic CJD. This finding has important potential implications as it raises the possibility that some humans infected with BSE prions may develop a clinical disease indistinguishable from classical CJD associated with type 2 PrPSc. This is, in our experience, the commonest molecular subtype of sporadic CJD. In this regard, it is of interest that the reported incidence of sporadic CJD has risen in the UK since the 1970s (Cousens et al., 1997). This has been attributed to improved case ascertainment, particularly as much of the rise is reported from elderly patients and similar rises in incidence were noted in other European countries without reported BSE (Will et al., 1998). However, it is now clear that BSE is present in many European countries, albeit at a much lower incidence than was seen in the UK. While improved ascertainment is likely to be a major factor in this rise, that some of these additional cases may be related to BSE exposure cannot be ruled out. It is of interest in this regard that a 2-fold increase in the reported incidence of sporadic CJD in 2001 has recently been reported for Switzerland, a country that had the highest incidence of cattle BSE in continental Europe between 1990 and 2002 (Glatzel et al., 2002). No epidemiological case–control studies with stratification of CJD cases by molecular subtype have yet been reported. It will be important to review the incidence of sporadic CJD associated with PrPSc type 2 and other molecular subtypes in both BSE-affected and unaffected countries in the future. (Collinge et al., 1995)
light of these findings. If human BSE prion infection can result in propagation of type 2 PrPSc, it would be expected that such cases would be indistinguishable on clinical, pathological and molecular criteria from classical CJD. It may also be expected that such prions would behave biologically like those isolated from humans with sporadic CJD with type 2 PrPSc. The transmission properties of prions associated with type 2 PrPSc from BSE-inoculated 129MM Tg35 mice are being investigated by serial passage.

We consider these data inconsistent with contamination of some of the 129MM Tg35 mice with sporadic CJD prions. These transmission studies were performed according to rigorous biosafety protocols for preparation of inocula and both the inoculation and care of mice, which are all uniquely identified by sub-cutaneous transponders. However, crucially, the same BSE inocula have been used on 129VV Tg152 and 129MM Tg45 mice, which are highly sensitive to sporadic CJD but in which such transmissions producing type 2 PrPSc were not observed. Furthermore, in an independent experiment, separate inbred lines of wild-type mice, which are highly resistant to sporadic CJD prions, also propagated two distinctive PrPSc types on challenge with either BSE or vCJD. No evidence of spontaneous prion disease or PrPSc has been seen in groups of uninoculated or mock-inoculated aged 129MM Tg35 mice.

While distinctive prion isolates have been derived from BSE passage in mice previously (designated 301C and 301V), these, in contrast to the data presented here, are propagated in mice expressing different prion proteins (Bruce et al., 1994). It is unclear whether our findings indicate the existence of more than one prion strain in individual cattle with BSE, with selection and preferential replication of distinct strains by different hosts, or that ‘mutation’ of a unitary BSE strain occurs in some types of host. Western blot analysis of single BSE isolates has not been reported.

Materials and methods

Generation of transgenic mice

The 759 bp human PrP ORF was amplified by PCR with pfu polymerase from genomic DNA encoding methionine at codon 129, using forward primer 5’-GTCGACCAGCTATGCGGAAACC-3’ and reverse primer 5’-CTCGAGAAAGCCCTCCTACTCCAC-3’. Restriction sites SalI and XhoI (underlined) were introduced in the forward and reverse primers, respectively, for cloning. The sequence was confirmed and ligated into the cosmid vector CosSHA-Tet (Scott et al., 1989). Microinjection of the purified DNA was carried out according to the standard protocol into single cell eggs of a strain of mice (FVB/N × Sv129 × C57BL/6) in which the murine PrP gene has been ablated (Bueler et al., 1992). Genotyping was performed by PCR, and PrP expression levels estimated by western blot analysis.

Transmission studies

Strict biosafety protocols were followed. Inocula were prepared, using disposable equipment for each inoculum, in a microbiological containment level 3 laboratory and inoculations performed within a class I microbiological safety cabinet. Five separate BSE inocula, each derived from single natural BSE-inoculated cows (B60, B62, 1064, 1066, 1783), and a separate inoculum prepared from a pool of five natural BSE brainstems (I038) were studied. Aliquots of these (except I783) have been used in previously published studies (Collinge et al., 1995; Hill et al., 1997). BSE tissues were collected under strict aseptic conditions using sterile instrumentation, specifically for transmission studies, by the UK Central Veterinary Laboratory (now the Veterinary Laboratories Agency (VLA)). The BSE pool homogenate was titrated into RIII wild-type mice at VLA with a resultant titre of 103.3 mouse intracerebral LD50 units/g of tissue. Sporadic and vCJD inocula were prepared from brain tissue from neuropathologically confirmed cases. Consent for use of tissues for research was obtained. The genotype of each transgenic mouse was confirmed by PCR of tail DNA prior to inclusion and all mice were uniquely identified by sub-cutaneous transponders. RIII/S/J mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and SJL/J OlaHsd mice were obtained from Harlan UK Ltd (Bicester, UK). Disposable cages were used, and all cage lids and water bottles were also uniquely identified by transponder and remained with each cage of mice throughout the incubation period. Care of the mice was according to institutional guidelines. Both transgenic and wild-type mice were anaesthetized with a mixture of halothane and O2, and intracranially inoculated into the right parietal lobe with 30 μl of a 1% brain homogenate prepared in PBS. Thereafter, all mice were examined daily for clinical signs of prion disease. Mice were killed if they were exhibiting any signs of distress or once a diagnosis of prion disease was established. Criteria for clinical diagnosis of scrapie in mice were as described previously (Carlson et al., 1986).

Neuropathology and immunohistochemistry

Mice were killed using CO2 asphyxiation, brains fixed in 10% buffered formal–saline and then immersed in 98% formic acid for 1 h and paraffin wax embedded. Serial sections of 4 μm were pre-treated with autolysing formic acid and 4 M guanidine thiocyanate. Abnormal PrP accumulation was examined using an anti-PrP monoclonal IgG antibody raised against recombinant human PrP (ICSM 35; A.Khalili-Shirazi, unpublished data), followed by a biotinylated anti-mouse IgG secondary antibody and an avidin–biotin–horseradish peroxidase conjugate before development with 3,3′-diaminobenzidine tetrachloride as the chromogen. The extent of gliosis was determined by GFAP (Dako) staining. Slides were pre-treated by heating in the microwave (900 W) in citrate buffer pH 6.0 for 25 min, followed by overnight incubation (1:1000). Biotinylated swine anti-rabbit

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immunoglobulins and avidin–biotin complex were applied as described above. Harris haematoxylin was used as the counterstain. Appropriate controls were used throughout.

**Western blotting**
Preparation of brain homogenates (10% w/v in PBS), proteinase K digestion (50 or 100 μg of proteinase K for 1 h at 37°C) and subsequent western blotting were performed as described previously (Wadsworth et al., 2001). For primary screening of both transgenic and wild-type mouse brain homogenates, blots were probed with a biotinylated anti-PrP monoclonal antibody which recognizes both human and mouse PrP (biotinylated-ICSM 55) in conjunction with an avidin–biotin–alkaline phosphatase conjugate (Dako) and development in chemiluminescent substrate (CDP-Star; Tropix Inc.). Primary screening of brain homogenates was performed blind to sample identity.

**Quantitation and analysis of PrP glycoforms**
Western blotting was performed as above but using different primary and secondary detection reagents. For transgenic mice expressing human PrP, blots were incubated with anti-PrP monoclonal antibody 3F4 (Kascak et al., 1998), whereas for wild-type mice expressing mouse PrP, blots were incubated with anti-PrP monoclonal antibody 6H4 (Prions, Switzerland), followed by incubation with goat anti-mouse IgG–alkaline phosphatase conjugate (Sigma) and development in chemiluminescent substrate (AttoPhos; Promega) and visualization on a Storm 840 PhosphorImager (Molecular Dynamics). Quantitation of PrP glycoforms was performed using ImageQuaNT software (Molecular Dynamics).

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**References**


