

## Evaluation of the Human Transmission Risk of an Atypical Bovine Spongiform Encephalopathy Prion Strain<sup>†</sup>

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**Bovine spongiform encephalopathy (BSE), the prion disease in cattle, was widely believed to be caused by only one strain, BSE-C. BSE-C causes the fatal prion disease named new variant Creutzfeldt-Jacob disease in humans. Two atypical BSE strains, bovine amyloidotic spongiform encephalopathy (BASE, also named BSE-L) and BSE-H, have been discovered in several countries since 2004; their transmissibility and phenotypes in humans are unknown. We investigated the infectivity and human phenotype of BASE strains by inoculating transgenic (Tg) mice expressing the human prion protein with brain homogenates from two BASE strain-infected cattle. Sixty percent of the inoculated Tg mice became infected after 20 to 22 months of incubation, a transmission rate higher than those reported for BSE-C. A quarter of BASE strain-infected Tg mice, but none of the Tg mice infected with prions causing a sporadic human prion disease, showed the presence of pathogenic prion protein isoforms in the spleen, indicating that the BASE prion is intrinsically lymphotropic. The pathological prion protein isoforms in BASE strain-infected humanized Tg mouse brains are different from those from the original cattle BASE or sporadic human prion disease. Minimal brain spongiosis and long incubation times are observed for the BASE strain-infected Tg mice. These results suggest that in humans, the BASE strain is a more virulent BSE strain and likely lymphotropic.**

Overwhelming evidence indicates that bovine spongiform encephalopathy (BSE), a prion disease that has been detected in several hundred thousand cattle in the United Kingdom and many other countries since the 1980s, has been transmitted to humans through the consumption of prion-contaminated beef, causing a prion disease named variant Creutzfeldt-Jacob disease (vCJD) (5, 19, 24). Over 200 cases of vCJD have been reported around the world (19). In 2004, two types of bovine prion disease that differ from the original BSE, now named classical BSE (BSE-C), were reported (3, 8). The two atypical BSE types were associated with prion protein (PrP) scrapie isoforms (PrP<sup>Sc</sup>) that after protease digestion, displayed distinct electrophoretic mobility or ratios of the PrP<sup>Sc</sup> glycoforms different from those of BSE-C (3, 8). Currently, a total of at least 36 cases of these two atypical BSE types have been reported for cattle older than 8 years (5; M. Caramelli, unpublished data). The two atypical BSE types are identified as BSE-H and bovine amyloidotic spongiform encephalopathy (BASE, also named BSE-L); the “L” and “H” identify the higher and lower electrophoretic positions, respectively, of their protease-resistant PrP<sup>Sc</sup> isoforms (7). The bovine pheno-

type and the PrP<sup>Sc</sup> molecular features of BASE have previously been described in detail (8). The histopathology of BASE and the PrP immunostaining pattern of BASE strains are characterized by the presence of prion amyloid plaques and a more rostral distribution of the PrP<sup>Sc</sup>, which at variance with BSE-C is present in the cerebral cortex, including the hippocampus, but is underrepresented in the brain stem (8). These phenotypic features and PrP<sup>Sc</sup> characteristics resemble a subtype of sporadic Creutzfeldt-Jacob disease (sCJD) named sCJDMV2, which affects subjects who are methionine (M)/valine (V) heterozygous at codon 129 of the PrP gene, and it is associated with PrP<sup>Sc</sup> identified as type 2 (15). This similarity has raised the question of whether sCJDMV2 is not sporadic but acquired from the consumption of BASE strain-contaminated meat (5, 8). To begin to investigate the transmissibility to humans and the “human” disease phenotype of BASE, including the involvement of the lymphoreticular system, we have inoculated brain homogenates from BASE-affected cattle to transgenic (Tg) mice expressing normal human PrP with Met at codon 129 (HuPrP-129M) in a mouse PrP-ablated background [Tg(HuPrP)] (13). The inoculated Tg mice were examined for attack rates and the disease phenotype, including the presence and characteristics of protease-resistant PrP<sup>Sc</sup> in the brain and spleen and the histopathology, along with the PrP<sup>Sc</sup> topography and pattern of deposition in the brain.

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### MATERIALS AND METHODS

**Transgenic mice.** Transgenic mice expressing human PrP-129M [Tg(HuPrP)] were reported previously (13). The Tg40 line that expresses human PrP-129M at

the wild-type level in the mouse PrP-ablated background was used in this study. Intracerebral (i.c.) inoculation of Tg mice and the monitoring of symptoms were performed as described previously (13). The mice were sacrificed 2 or 3 days after the appearance of symptoms or at death, and the brains and spleens were taken. The brains were sliced sagittally, with half frozen for immunochemical studies and the other half either fixed in formalin for histological and immunohistochemical staining or frozen for histoblot analysis (see below). Total PrP as well as proteinase K (PK)-resistant PrP<sup>Sc</sup> was determined by immunoblotting in sodium dodecyl sulfate (SDS)-polyacrylamide gels as described below. This study was conducted with approvals from the Institutional Review Board and the Institutional Animal Care and Use Committee.

**Immunoblotting, histology, histoblotting, and immunohistochemistry.** Frozen brain or spleen tissues were homogenized in 2 volumes of cold phosphate-buffered saline to obtain 33% (wt/vol) crude homogenate for storage in aliquots at  $-80^{\circ}\text{C}$ . The frozen 33% crude homogenate was thawed at  $4^{\circ}\text{C}$  for 2 h and diluted to 10% (wt/vol) with the lysis buffer (final concentration, 100 mM Tris, 10 mM EDTA, 100 mM NaCl, 0.5% sodium deoxycholate, 1.0% NP-40, pH 8.0). After incubation at room temperature for 15 min, the 10% homogenate was subjected to sonication with the Ultrasonic Dismembrator 100 (Fisher Scientific) for 3 min. The sonicated 10% homogenate was treated with 100  $\mu\text{g}/\text{ml}$  PK (Roche Diagnostics GmbH, Mannheim, Germany) for 30 min at  $37^{\circ}\text{C}$  and denatured by being boiled at  $100^{\circ}\text{C}$  for 10 min after being mixed with an equal volume of  $2\times$  sample buffer (200 mM Tris-HCl, pH 6.8, 2% SDS, 40% glycerol, 0.04% Coomassie blue G-250, 2%  $\beta$ -mercaptoethanol). The enrichment of PrP<sup>Sc</sup> by precipitation with sodium phosphotungstate (NaPTA) was performed virtually as previously reported (18), and special care and efforts were taken to ensure that the pellets were completely resuspended each time. Proteins were separated by precast 10 to 20% gradient Tris-Tricine gel (Bio-Rad), transferred to a polyvinylidene difluoride membrane, and subjected to Western blot analysis with monoclonal antibody (MAb) 8H4, 6H4, or 3F4 in conjunction with horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin G Fc antibody (GE Healthcare, Buckinghamshire, United Kingdom) as described previously (13). The blots were developed with the ECL Western blotting detection reagent (GE Healthcare Amersham, Buckinghamshire, United Kingdom) and exposed to X-ray films. The blots were digitized by scanning the film. To determine the precise molecular weights of the bands, the digitized blots were analyzed by image acquisition and analysis software (UVP, Upland, CA) that automatically detects the midpoint of the band and calculates the molecular weight based on the sizes of the unglycosylated PK-resistant PrP fragments of sCJDMM1 and sCJDMM2; the values were statistically analyzed by Matlab 7.0 software (MathWorks, Natick, MA). To determine the glycoform ratios of PK-resistant PrP<sup>Sc</sup> fragments, each PrP band on the digitized blots was quantified with UN-SCAN-IT software (Silk Scientific, Orem, UT); the values from duplicate blots were analyzed with Excel software to calculate the averages and standard deviations and to create the column chart.

Histological staining with hematoxylin and eosin (H&E) and immunohistochemical staining with 3F4 were performed as reported previously (13). Histoblot analysis was performed mostly as described previously (20), with the following modifications: the cryosections were 12  $\mu\text{m}$  thick, and the sections were treated with 100  $\mu\text{g}/\text{ml}$  of proteinase K for 4 h at  $37^{\circ}\text{C}$ , incubated with monoclonal antibody 3F4 (1:10,000 dilution) overnight at  $4^{\circ}\text{C}$ , followed by incubation with alkaline phosphatase-conjugated goat anti-mouse secondary antibody (1:500; DAKO), and developed with BCIP (5-bromo-4-chloro-3-indolylphosphate)-nitroblue tetrazolium solutions (Sigma).

## RESULTS

To assess the transmissibility of BASE in humans, two BASE isolates (8) were used to intracerebrally inoculate 30 Tg40 mice that express normal levels of human PrP-129M. More than half of the inoculated mice (18/30) became infected, as determined by the presence of protease-resistant PrP<sup>Sc</sup>, with average incubation times of  $649 \pm 34$  days for BASE isolate 1 and  $595 \pm 28$  days for BASE isolate 2, respectively (Table 1). Ten of the 18 infected mice that could be examined showed clear clinical signs of disease (Table 1), including hunched backs, ruffled fur, lethargy, occasional wobbling, and rigid tails. These signs were best detected in the younger mice, because in mice older than 24 months, the signs became difficult to distinguish from aging-related changes.

TABLE 1. BASE transmission in Tg(HuPrP) mice

Inoculum	Attack rate as determined by:			Incubation time (days)
	Clinical signs	Presence of PrP <sup>Sc</sup>	Spongiform degeneration	
BASE-1	4/15	9/15	1 (focal)/8	$649 \pm 34$
BASE-2	6/15	9/15	1 (focal)/11	$595 \pm 28$
sCJDMM1	10/10	9/10	4/4	$263 \pm 13^a$
sCJDMM2	9/9	9/9	7/7	$267 \pm 17$

<sup>a</sup> Reported previously (13).

All the Tg40 mice were examined for the presence of PK-resistant PrP<sup>Sc</sup> in the brain by immunoblot analysis both directly and after enrichment with NaPTA precipitation. Such immunoblot analysis with three monoclonal antibodies (3F4, 6H4, and 8H4) to various PrP regions (12, 14, 25) showed that all 18 BASE strain-infected Tg40 mice accumulated comparable amounts of PK-resistant PrP<sup>Sc</sup> in the brain (Fig. 1A, Table 1, and data not shown). The electrophoretic mobility of PK-resistant PrP<sup>Sc</sup> fragments from all the BASE strain-infected Tg40 mice was indistinguishable from that of the PK-resistant PrP<sup>Sc</sup> present in either the BASE strain inoculum or sCJDMM2, which contains type 2 PrP<sup>Sc</sup> (Fig. 1A). The PK-resistant PrP<sup>Sc</sup> fragments associated with both the BASE strain-infected Tg40 mice and the BASE isolates migrated slightly faster than those of BSE-C as originally reported (8). Measurements with software that automatically calculates the midpoint of the bands revealed a difference of  $0.29 \pm 0.12$  kDa in gel mobility between the unglycosylated PK-resistant PrP<sup>Sc</sup> bands of the BASE strain (native as well as from the Tg40 mice) and BSE-C.

The glycoform ratio of PrP<sup>Sc</sup> in isolates from the BASE strain-infected Tg40 mice was slightly different from that of the BASE isolates (Fig. 1B), and both were quite different from that of BSE-C (Fig. 1B). The monoglycosylated form was the most prominent species in the BASE strain inocula, where the glycoform ratio (diglycosylated-to-monoglycosylated-to-unglycosylated) is 32:41:27, whereas the diglycosylated form was slightly more intense than the monoglycosylated form in BASE strain-infected Tg40 mice, where the glycoform ratio is 44:39:17 (Fig. 1B). In contrast, the diglycosylated form accounted for over 70% of the total PrP<sup>Sc</sup> in BSE-C (glycoform ratio of 72:20:8).

PrP<sup>Sc</sup> in the spleen was also examined after NaPTA enrichment for all 30 BASE strain-inoculated Tg40 mice. PK-resistant PrP<sup>Sc</sup> was readily detected in the spleens of four mice (Fig. 1C), all of which also contained PK-resistant PrP<sup>Sc</sup> in the brain. The electrophoretic mobility of the spleen PrP<sup>Sc</sup> was similar to that of the brain PrP<sup>Sc</sup>. The glycoform ratio of the spleen PrP<sup>Sc</sup> was different from that of the brain and was characterized by the prominence of the monoglycosylated and unglycosylated forms (Fig. 1C), but the glycoform ratio may have been affected by the NaPTA enrichment. In contrast, none of the nine Tg40 mice inoculated with sCJDMM1 had detectable PK-resistant PrP<sup>Sc</sup> in the spleen after NaPTA enrichment (data not shown).

None of the 12 BASE strain-infected Tg40 mice examined showed prominent and consistent histopathological changes related to prion diseases (Fig. 2A). Focal, ambiguous spongiform degeneration was observed for two mice. No PrP amyloid plaques were observed in BASE strain-infected Tg40 mice.

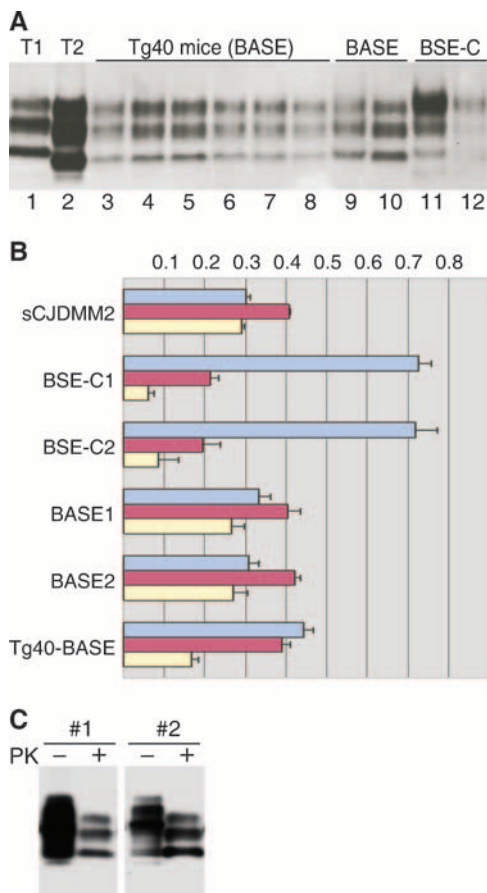


FIG. 1. Immunoblots and glycoform ratios of PK-resistant PrP<sup>Sc</sup> from sCJD-affected, BASE strain-infected Tg(HuPrP) mice, the BASE strain inocula, and BSE-C and of PK-resistant PrP<sup>Sc</sup> from the spleens of BASE strain-infected Tg(HuPrP) mice. (A) Immunoblot of PK-resistant PrP<sup>Sc</sup> in the brain. Lanes 1 and 2, type 1 (sCJDMM1) (T1) and type 2 (sCJDMM2) (T2) sCJD, respectively; lanes 3 to 6, Tg(HuPrP) (Tg40) mice infected with BASE isolate 1 inoculum; lanes 7 to 8, Tg40 mice infected with BASE isolate 2 inoculum; lane 9, BASE isolate 1; lane 10, BASE isolate 2; lanes 11 and 12, two BSE-C isolates. All brain homogenates were treated with 100 µg/ml of PK for 30 min at 37°C and processed for immunoblot analysis with MAb 8H4. Five microliters of 10% brain homogenate was loaded for lanes 3 to 10. (B) Glycoform ratios of PK-resistant PrP<sup>Sc</sup> in the brain. The upper (diglycosylated) (blue), middle (mostly monoglycosylated) (red), and lower (unglycosylated) (yellow) bands of PK-resistant PrP<sup>Sc</sup> from BASE strain-infected Tg40 mice, the BASE strain, and BSE-C were quantified after optical scanning of duplicate immunoblots for panel A. Error bars indicate standard deviations. (C) PK-resistant PrP<sup>Sc</sup> in the spleen. Ten milligrams of spleen tissue each from two of the BASE strain-infected Tg(HuPrP) (Tg40) mice (#1 and #2) was homogenized, PrP<sup>Sc</sup> enriched by NaPTA precipitation, and either treated (+) or not treated (-) with 100 µg/ml of PK for 30 min at 37°C, followed by electrophoresis in a 10 to 20% Tris-Tricine SDS-polyacrylamide gradient gel and immunoblot analysis with MAb 8H4.

Histoblot analysis with MAb 3F4 showed a very distinct and selective distribution of PrP<sup>Sc</sup> (Fig. 3A to D). Particular nuclei or groups of adjacent periventricular nuclei in the thalamus, hypothalamus, and brain stem were intensely immunostained for PrP<sup>Sc</sup> (Fig. 3B to D). In contrast, PrP<sup>Sc</sup> appeared to be overall less intense in the cerebral and cerebellar cortices (Fig. 3A to D). Immunohistochemical staining of paraffin-embed-

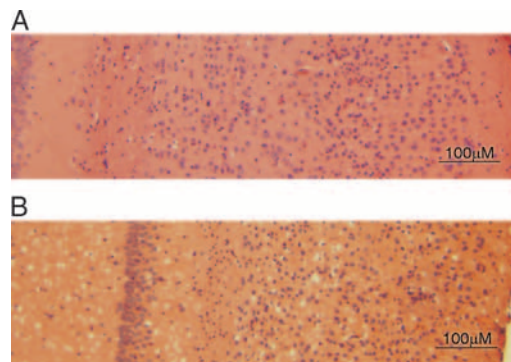


FIG. 2. Histopathology (with H&E) of BASE strain-infected and sCJDMM1-infected Tg(HuPrP) mice. (A) No consistent pathology was detected in the cerebral cortex as well as subcortical brain regions of symptomatic and immunoblot-positive BASE strain-infected Tg(HuPrP) (Tg40) mice. (B) In contrast, Tg40 mice inoculated with sCJDMM1 brain homogenate showed widespread spongiform degeneration.

ded brain tissue with 3F4 revealed PrP deposits in 5 of the 11 BASE strain-infected Tg40 mice examined. PrP<sup>Sc</sup> deposits that stained intensely in the histoblots consisted of relatively large and well-circumscribed granules (Fig. 3E and G). Fine granular or small plaque-like aggregate patterns were occasionally seen in inferior regions of the cerebral cortex and in the thalamus (Fig. 3I and data not shown). In contrast, widespread, mostly fine-granular staining was detected in the cerebral cortex of symptomatic Tg40 mice inoculated with sCJDMM1 brain homogenate (Fig. 3J).

The histopathological features of the BASE strain-inoculated Tg40 mice were quite different from those observed following inoculation with brain homogenates from the two forms of sCJD, sCJDMM1 and sCJDMM2. The sCJDMM1-inoculated Tg40 mice had widespread spongiform degeneration in the cerebrum (Fig. 2B) and moderate apoptosis of neuronal cells without spongiform degeneration in the cerebellum (13). Widespread spongiform degeneration was also seen in Tg40 mice inoculated with sCJDMM2 brain homogenate (data not shown).

### DISCUSSION

We have shown that 60% of our Tg40 mice (in an inbred FVB background) that express normal levels of human PrP-129M became infected 20 to 22 months after i.c. inoculation with 0.3 mg of brain tissue from the two BASE isolates, suggesting a titer of approximately 3 50% infective dose units per milligram of brain tissue in the Tg40 line. An approximately 20% attack rate has been reported for the Tg650 line (in a mixed 129/Sv × C57BL/6 background) after i.c. inoculation with 2 mg brain tissues from BSE-C-infected cattle (2). It is noteworthy that the Tg650 mice express human PrP-129M at five to eight times the normal level, and high PrP levels are known to increase prion transmissibility (9, 17, 22). Inefficient BSE-C transmissions (0 to 30%) in Tg mouse lines of other genetic backgrounds expressing human PrP-129M at one or two times the normal level have also been reported by different groups (1, 4). Although it is difficult to compare results from different mouse lines, these findings suggest that the BASE strain has higher transmissibility than BSE-C does for human-



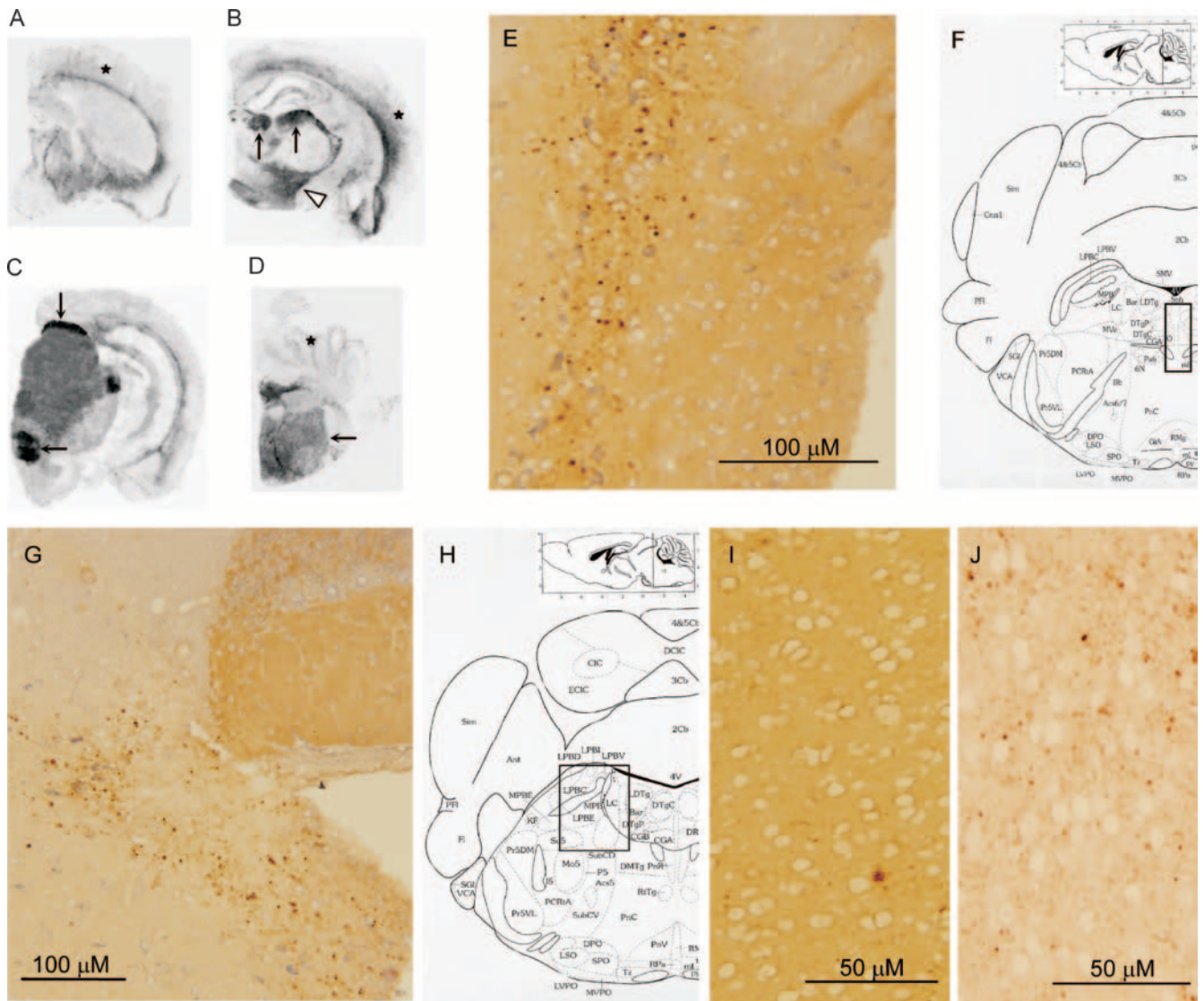


FIG. 3. Histoblot analysis and immunohistochemistry of BASE strain-infected and sCJDMM1-infected Tg(HuPrP) mice. (A to D) The histoblot analysis revealed preferential immunostaining of the PrP<sup>Sc</sup> in the dorsal thalamic nuclei (arrows in panel B), along with hypothalamic nuclei (arrowhead) and brain stem nuclei (arrows in panels C and D), while PrP<sup>Sc</sup> in the cerebral and cerebellar cortices (stars in panels A, B, and D) was mostly limited to the deep and inferior cortical regions. (E to J) The PrP immunostaining (E and G) of the intensely PrP-reactive brain stem nuclei in histoblot analysis (boxed regions in panels F and H) revealed coarse PrP granules, while the PrP immunostain in the cerebral cortex (I) was minimal and characterized mostly by a plaque-like pattern. In contrast, widespread fine-granular PrP immunostaining was observed in the cerebral cortex of symptomatic Tg40 mice following inoculation of sCJDMM1 brain homogenates (J). Monoclonal antibody 3F4 was used for all the staining.

ized Tg mice with PrP-129M and possibly for humans with PrP-129MM. The BASE strain also appears to be more virulent than BSE-C in bovinized Tg mice, since the incubation time for the BASE strain is  $185 \pm 12$  days, whereas that for BSE-C is  $230 \pm 7$  days (7). Nevertheless, compared with the 100% attack rate and incubation times of ~9 months for sCJDMM1 and sCJDMM2 in the Tg40 line (Table 1), the 60% attack rate and unusually long incubation times (20 to 22 months) for the BASE strain in the same Tg line suggest that the transmission barrier from the BASE strain to humans with PrP-129MM is still quite significant.

PK-resistant PrP<sup>Sc</sup> was also detected in the spleen in 4 out of 18 BASE strain-infected Tg40 mice. In contrast, no spleen

involvement could be demonstrated for the Tg40 mice following i.c. inoculation with human PrP<sup>Sc</sup> from sCJDMM1. This is the first report of the presence of PrP<sup>Sc</sup> in the spleens of humanized Tg mice after i.c. inoculation with a BSE strain, suggesting that the BASE strain, like BSE-C, where at least in vCJD-infected subjects PrP<sup>Sc</sup> and prion infectivity have been detected in spleens and tonsils (6, 11), is intrinsically lymphotropic. Therefore, lymphoid tissues of BASE strain-infected individuals might also carry prion infectivity.

The gel mobility of the PK-resistant PrP<sup>Sc</sup> recovered from the BASE strain-inoculated Tg40 mice was consistently slightly faster than the mobility of BSE-C, as originally reported for the BASE strain (8). The computed difference in gel mobilities

between BASE and BSE-C PrP<sup>Sc</sup> is  $0.29 \pm 0.12$  kDa, corresponding to 2 to 4 amino acid residues. In contrast, the gel mobilities of the PK-resistant PrP<sup>Sc</sup> species from the BASE strain, BASE strain-infected Tg40 mice, and sCJDMM2, which was used as representative of human PrP<sup>Sc</sup> of type 2, were indistinguishable. This finding suggests that the PK-resistant PrP<sup>Sc</sup> electrophoretic heterogeneity between the BASE strain and BSE-C falls well within the 7-amino-acid variability of the N terminus (positions 92 to 99) that is consistently found in PK-resistant PrP<sup>Sc</sup> of type 2 (16). Therefore, despite their minor but distinct variability in gel mobility, both the BASE strain and BSE-C PrP<sup>Sc</sup> species appear to belong to the PrP<sup>Sc</sup> of type 2. However, the PrP<sup>Sc</sup> glycoform ratios of BASE strain-infected Tg40 mice and the BASE strain inocula display a small but statistically significant difference (Fig. 1). Therefore, PrP<sup>Sc</sup> in BASE strain-infected human subjects may be expected to display a different glycoform ratio from that of the BASE strain. It is worth noting that the electrophoretic characteristics of the PK-resistant PrP<sup>Sc</sup> of some human prion strains has been faithfully reproduced by our Tg40 line as well as by other humanized mouse lines (10, 13, 21).

Two distinct histopathological and PrP immunohistochemical phenotypes have been reported following BSE-C inoculation: one reproduced the distinctive features of vCJD with the "florid" plaques that intensely immunostained for PrP, and the other was reminiscent of sCJDMM1, with prominent spongiform degeneration and no plaque PrP immunostaining (1, 23). The brain histopathology, the PrP<sup>Sc</sup> distribution, and the PrP immunostaining pattern of BASE strain-inoculated Tg40 mice were definitely distinct from such features described above (1, 23), further supporting the notion that BASE and classical BSE are associated with two distinct prion strains (8).

The relatively easy transmission of BASE to humanized Tg mice indicates that effective cattle prion surveillance should be maintained until the extent and origin of this and other atypical forms of BSE are fully understood.

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