Neurobiology of Disease

Bovine Spongiform Encephalopathy Induces Misfolding of Alleged Prion-Resistant Species Cellular Prion Protein without Altering Its Pathobiological Features

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Bovine spongiform encephalopathy (BSE) prions were responsible for an unforeseen epizootic in cattle which had a vast social, economic, and public health impact. This was primarily because BSE prions were found to be transmissible to humans. Other species were also susceptible to BSE either by natural infection (e.g., felids, caprids) or in experimental settings (e.g., sheep, mice). However, certain species closely related to humans, such as canids and leporids, were apparently resistant to BSE. *In vitro* prion amplification techniques (saP-MCA) were used to successfully misfold the cellular prion protein (PrP^c) of these allegedly resistant species into a BSE-type prion protein. The biochemical and biological properties of the new prions generated *in vitro* after seeding rabbit and dog brain homogenates with classical BSE were studied. Pathobiological features of the resultant prion strains were determined after their inoculation into transgenic mice expressing bovine and human PrP^C. Strain characteristics of the *in vitro*-adapted rabbit and dog BSE agent remained invariable with respect to the original cattle BSE prion, suggesting that the naturally low susceptibility of rabbits and dogs to prion infections should not alter their zoonotic potential if these animals became infected with BSE. This study provides a sound basis for risk assessment regarding prion diseases in purportedly resistant species.

Introduction

Bovine spongiform encephalopathy (BSE) was first described in cattle in 1985 (Wells et al., 1987) and became a major public health concern when evidence linked it to variant Creutzfeldt-Jakob disease in humans (Bruce et al., 1997; Hill et al., 1997). Unlike other animal prion diseases, BSE has transmitted naturally to humans and also to other domestic species including goats (Eloit et al., 2005), cats (Aldhous, 1990; Wyatt et al., 1991), zoological-kept felidae (Willoughby et al., 1992; Lezmi et al., 2003; Bencsik et al., 2009; Eiden et al., 2010), and ruminants

(Kirkwood and Cunningham, 1994). Experimentally, sheep are susceptible to BSE (Foster et al., 1993; Bellworthy et al., 2008) as are many laboratory animals including primates, pigs, mice, and guinea pigs (Fraser et al., 1988; Lasmezas et al., 1996; Wells et al., 2003; Castilla et al., 2004; Konold et al., 2009; Safar et al., 2011).

Most BSE cases diagnosed in cattle have been classified as classical BSE, which was believed to be caused by a single stable agent (Wells and Wilesmith, 1995). BSE exhibits a unique biochemical "signature", which has solely been associated with this particular prion strain, has been observed in the vast majority of BSE cases in cattle and is maintained when transmitted to other susceptible species (Collinge et al., 1996; Stack et al., 2002; Biacabe et al., 2004).

Even though BSE has crossed many transmission barriers, other species which have presumably been exposed to BSE prions have not been documented to succumb to prion disease. Among these are the members of the canidae and leporidae families (Gibbs and Gajdusek, 1973; Barlow and Rennie, 1976; Vorberg et al., 2003). The lack of literature in these species with respect to natural or experimental TSE transmissions supported their categorization as purportedly prion-resistant. However, recently developed *in vitro* prion amplification techniques, such as protein misfolding cyclic amplification (PMCA) (Saborio et al., 2001)

and its variants (Castilla et al., 2006), are allowing reclassification of the strength of certain species transmission barriers (Fernandez-Borges et al., 2009; Chianini et al., 2012). The normal cellular prion protein (PrP^C) from these purportedly resistant species can be successfully misfolded *in vitro*. However, it is essential to determine whether these novel abnormal prions are capable of inducing disease and whether their strain properties are maintained or not after passage through a supposedly resistant species. Due to its ability to infect humans, prediction of the behavior of BSE in purportedly resistant species that are either consumed by or in close contact with humans is vital for risk assessment. The design of experiments involving the natural, long-lived hosts is frequently unaffordable. Therefore, the use of repeated passages of intracerebral inoculation of transgenic mouse models (overexpressing the PrP^C of the species under investigation) are used to overcome these limitations (Castilla et al., 2004; Sigurdson et al., 2006). Furthermore, an *in vitro* approach in which brain homogenates from the chosen species under investigation are submitted to several rounds of amplification by PMCA greatly increases transmission efficiency (Castilla et al., 2008; Chianini et al., 2012).

In this study, we analyzed the biochemical and biological properties of new prions generated *in vitro* after seeding rabbit and dog brain homogenates with classical BSE. Pathobiological features of the resultant prion strains were studied after their inoculation in transgenic mice expressing bovine and human PrP^C. Strain characteristics of the *in vitro*-adapted rabbit and dog BSE agent remained invariable with respect to the original cattle BSE prion, suggesting that the naturally low susceptibility of rabbits and apparently of dogs to prion infections (Polymenidou et al., 2008; Kurt et al., 2011) should not alter their zoonotic potential if these animals became infected with BSE. This study provides a sound basis for risk assessment regarding prion diseases in purportedly resistant species.

Materials and Methods

Inocula preparation for in vitro *prion replication studies.* Brain homogenates (10^{-1} in PBS) for use as seeds were prepared by manual potter from a brain of a clinically affected BSE-positive bovid (TSE/08/59) supplied by the Veterinary Laboratory Agency (New Haw, Addlestone, Surrey, UK). The titer of this inoculum was ~10^8 ID50 units per gram of bovine brainstem, as determined in the boTg110 mouse line (Castilla et al., 2003).

Generation of in vitro *PrP^{res} by serial automated PMCA.* The *in vitro* prion replication, including the PrP^{Sc} detection of amplified samples, was performed as described previously with minor modifications (Saá et al., 2006). Briefly, 5 µl aliquots of 10%, and 50% in the case of the dog, brain homogenate from animals infected by BSE were diluted in 50 µl of 10% normal rabbit and dog brain homogenates and loaded onto 0.2 ml PCR tubes. Rabbit and dog brains used for substrate were previously perfused using PBS + 5 mM EDTA. The blood-depleted brains were frozen immediately before preparing the 10% brain homogenates (PBS + NaCl 1% + 1% Triton X-100). Tubes were positioned on an adaptor placed on the plate holder of a microsonicator (Misonix, Model 4000) and subjected to cycles of 30 min incubation at 38°C followed by a 20 s pulse of sonication at potency 80. Samples were incubated in the water bath of the sonicator without shaking. Serial rounds of PMCA consisted of 48 h of standard PMCA followed by serial *in vitro* 1:10 passages in fresh rabbit brain substrate. An equivalent number of unseeded tubes containing the corresponding brain substrate were subjected to the same number of rounds of serial automated PMCA (saPMCA) to control cross-contamination and/or the generation of spontaneous PrP^{res}. The detailed protocol for PMCA, including reagents, solutions, and troubleshooting, were previously published (Saá et al., 2005).

Inocula preparation for in vivo *prion replication studies.* Inocula for second passages where prepared by homogenizing the harvested CNS (spinal cord and olfactory bulb) material from infected first passage mice 1:10 (w/v) with sterile PBS. The homogenates where filtered through a surgical gauze. Homogenate from one mouse brain from the first passage was used to prepare the inocula, the mice used had 365 d postinoculation (dpi) in the case of BSE-DoPrP^{res} and 312 in the case of BSE-RaPrP^{res}.

Biochemical characterization of in vitro-*generated and* in vivo-*generated prion strains.* The standard procedure to digest PrP^{Sc} was performed following the basic conditions described previously (Castilla et al., 2005a). Briefly, PMCA samples were digested using 85–200 µg/ml protease K (PK) during 1 h at 42°C with shaking (450 rpm). Digestion was stopped by adding electrophoresis loading buffer and the samples were analyzed by Western blotting.

tgBov and tgHum mice inoculation. The bovine *PRNP* expression mouse model *boTg110* (*tgBov*) was established and characterized as described previously (Castilla et al., 2003; Castilla et al., 2005b; Espinosa et al., 2007). Briefly, these mice express bovine PrP^C under the murine *PRNP* promoter in a murine PrP$^{0/0}$ background. Bovine PrP^C expression levels in this mouse line are stated to be eightfold higher than the PrP^C levels found in cattle brain homogenates. As a model to assess the theoretical human susceptibility, the model *Tg340* (*tgHum*), which expresses human *PRNP* (4-fold higher than in human brain) with a methionine at codon 129 (*Met129*) also on a murine *PRNP*-null background, was used (Padilla et al., 2011).

Each 6- to 8-week-old mouse received an intracerebral inoculation (20 µl) through the parietal bone using a 50 µl SGC precision syringe, a 25 gauge needle, and a repeatability adaptor. During the inoculation procedure, the mice were kept under deep gaseous anesthesia (isoflurane). A subcutaneous dose of buprenorphine was administered before awakening to minimize postinoculation pain.

Mice were kept under controlled conditions at a room temperature of 21–22°C, 12 h light/dark cycle and 60% relative humidity. Cages were in HEPA-filtered (both air inflow and extraction) ventilated racks. The mice were fed *ad libitum*. To evaluate transmissible spongiform encephalopathy (TSE)-related clinical signs, mice were observed daily and their neurological status was assessed twice a week. The presence of three signs of neurological dysfunction (using 10 different criteria) (Scott et al., 1989) was necessary for a mouse to score positive for prion disease. Only female mice were used to avoid male aggression.

In vitro inocula (BSE-RabPrP^{res} and BSE-DogPrP^{res}) were subjected to at least 15 rounds of saPMCA (a 10^{-15} dilution of the initial seed) after the first evidence of *in vitro* crossing of the species barrier (Fig. 1).

The groups were designed as follows: for the *boTg110* model 10 animals were inoculated with cattle BSE on first passage and 9 on second passage; 16 animals were inoculated with BSE-RabPrP^{res} on first passage and 12 on second; 17 animals were inoculated with BSE-DogPrP^{res} on first passage and 13 on second, and finally, as a negative control 12 mice where inoculated with brain homogenate from a clinically healthy, BSE-negative cow. For the *Tg340* (*Met129*) model, 10 animals were inoculated with cattle-BSE, 9 with BSE-RabPrP^{res}, and 6 with BSE-DogPrP^{res}.

Ethics statement. The procedures involving animals were approved by the animal experimentation ethics committee of the Autonomous University of Barcelona (Reference No. 585-3487) in agreement with Article 28, sections (a), (b), (c), and (d) of the "Real Decreto 214/1997 de 30 de Julio" and the European Directive 86/609/CEE and the European council Guidelines included in the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes.

Sample processing and general procedures. Immediately after mice were killed (by intraperitoneal overdose of pentobarbital and decapitation) the brain was extracted and placed into 10% phosphate buffered formalin. Transversal sections of the brain were obtained at the level of the optic chiasm, piriform cortex, and medulla oblongata. Samples were dehydrated through increasing alcohol concentrations and xylene before embedding in paraffin wax. Four-micrometer sections were cut and mounted on glass microscope slides and stained with hematoxylin and eosin for morphological evaluation. Further slides were mounted in 3-trietoxysilil-propilamine-coated glass slides for immunohistochemical studies.

Immunohistochemistry. Immunohistochemistry (IHC) against PrP^d was performed as previously described (Siso et al., 2004). Briefly, depar-

Rounds (R1-R10) of serial automated PMCA using rabbit, dog and cattle brain homogenate as substrates.

Seed	% inoculum	Substrate	Dilution	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10
BSE	10%	Rabbit	1:10	·	·	·	□	□	□	■	■	■	■
Unseeded		Rabbit	1:10	□	□	□	□	□	□	□	□	□	□
BSE	10%	Dog	1:10	·	·	□	□	□	□	□	□	□	□
BSE	50%	Dog	1:2	·	·	□	□	▨	▨	■	■	■	■
Unseeded		Dog	1:10	□	□	□	□	□	□	□	□	□	□
BSE	10%	Cattle	1:10	·	·	■	■	■	■	■	■	■	■
Unseeded		Cattle	1:10	□	□	□	□	□	□	□	□	□	□

The gray scale in the boxes indicates the % of positive tubes (showing PK resistant rabbit, dog and cattle PrP^{res}) out of the total number of tubes sonicated (n=4).

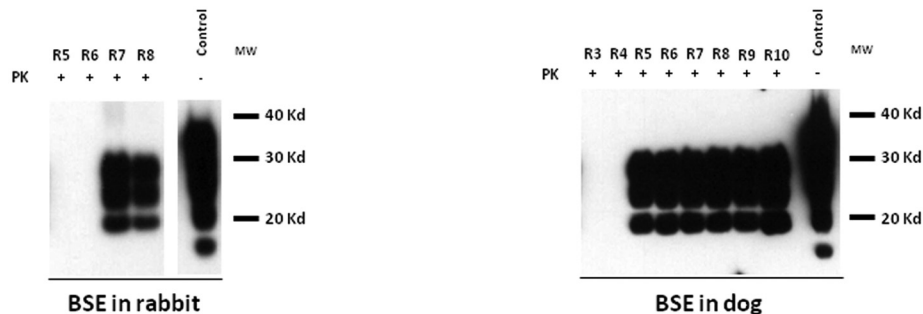· Not determined   □ 0%   ▢ 25%   ▨ 50%   ▩ 75%   ■ 100%

Figure 1. *In vitro* amplification experiments. Rounds (R1-R10) of serial automated PMCA using rabbit, dog, and cattle brain homogenate as substrate. Note dog brain inocula are at 10 and 50%. Serial rounds (R5-R8 for rabbit and R3-R10 for dog) were selected to show biochemical analyses of BSE seeded PrP^{res} generated *in vitro* by saPMCA. Samples from one of four tubes subjected to saPMCA were digested with 100 µg/ml PK and analyzed by Western blot using monoclonal antibody D18. Control, Undigested cattle brain homogenate; R, round.

affinized sections were immersed in formic acid and boiled at low pH in a pressure cooker, with endogenous peroxidases blocked. After pretreatment with PK, the sections were incubated overnight with primary anti-PrP mAb 6H4 antibody (1:2000, kindly provided by Prionics AG), and finally, visualized using the Dako EnVision system and 3,3′-diaminobenzidine as the chromogen substrate as per the manufacturer's instructions. As a background control, the primary antibody incubation was omitted. No labeling was observed in any of the control slides.

Semiquantification and data analysis. Semiquantification of the histopathological lesions and PrP^d immunolabeling was performed. Subjective scores ranging from 0 (absence of spongiosis or immunolabeling) to 5 (maximum intensity of lesion or immunolabeling) were assigned to each brain area studied and profiles were constructed. Intermediate levels were (1) mild, (2) moderate, (3) marked, and (4) intense. Each area was investigated as a global region for the score.

The brain profiling approach consisted of a systematic screening of the different brain regions to obtain comparable data from the different prions used to challenge mice. A total of 15 different regions were chosen and semiquantitatively evaluated by eye as a whole for labeling intensity. The results were plotted on a graph that summarizes the distribution of lesions and PrP^d throughout the brain. This allowed for comparison of the different groups studied.

For both parameters, results were plotted as a function of the anatomical area. Areas were ordered along the *x*-axis in an attempt to represent the caudorostral axis of the brain. This methodology was adapted from a previous study performed on BSE-infected cattle (Vidal et al., 2005). For statistical analysis, the Mann–Whitney *U* test for nonparametric variables was applied (*$p < 0.05$ with 95% confidence interval, **$p < 0.01$

with 99% confidence interval). Graphs were plotted using Microsoft Office 2007 Excel software.

Results

In vitro generation of rabbit and dog adapted BSE prions

We seeded normal rabbit and dog brain homogenates *in vitro* with cattle BSE brain homogenate before saPMCA in an attempt to study the ability to generate rabbit and dog adapted BSE PrP^{res} (BSE-RaPrP^{res} and BSE-DoPrP^{res}, respectively). Although saPMCA is not a quantitative technique, rabbit PrP^C appeared to be more susceptible to protein misfolding as BSE-seeded rabbit brain homogenate generated PK resistant RaPrP^{res} by round 7 (Fernández-Borges et al., 2009; Chianini et al., 2012) compared with dog PrP^C which showed higher resistance (Fig. 1). This impaired ability to convert dog PrP^C was consistent in all the seeds used (our unpublished observations) suggesting that *in vitro* propagation was impeded. Because the *in vitro* crossing of species barriers is a quasi-stochastic phenomenon, we modified the settings of saPMCA in an attempt to adapt it to the potentially most resistant species barrier known in mammals. Thus, dog brain homogenates were seeded with a larger amount of BSE-positive cattle brain material (50% instead of the standard 10%) and the dilutions were performed at 1:2 (v/v) during subsequent rounds of PMCA. These changes enabled the cattle-dog transmission barrier to be overcome at round 5 (Fig. 1). Biochemical characterization, by Western blotting, of the *in vitro* generated
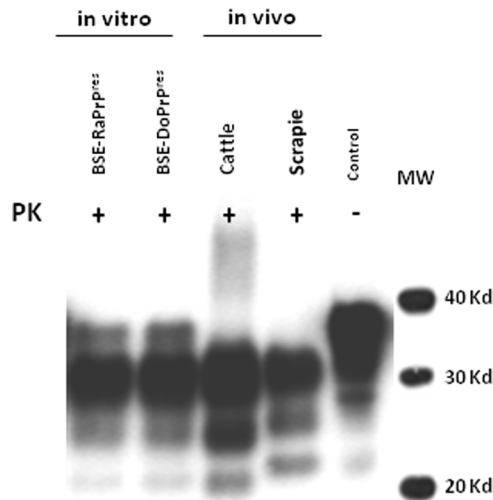
Figure 2. Biochemical analyses of BSE seeded PrP^res generated *in vitro* by saPMCA using cattle, rabbit, and dog brain homogenates as substrates. Cattle, rabbit, and dog brain homogenates seeded with BSE-positive cattle brain homogenate were subjected to saPMCA. Seeded samples (BSE-BoPrP^res, BSE-RaPrP^res, and BSE-DoPrP^res) from round 10 were digested with 100 μg/ml PK and analyzed by Western blot using monoclonal antibody D18. The electrophoretic migration patterns in all the *in vitro* samples and the *in vivo* BSE-positive cattle sample used as positive control were indistinguishable. Scrapie was used as reference for determining the different electrophoretic migration patterns. Control, Undigested cattle brain homogenate.

Table 1. Attack rates and mean survival times (±SEM) of inoculated *tgBov* mice

	First passage		Second passage	
	Attack rate	Survival time (dpi) (±SEM)	Attack rate	Survival time (dpi) (±SEM)
Cattle BSE	10/10	355 (±15)	9/9	348 (±11)
BSE-RaPrP^res	16/16	395 (±16)	11/12[a]	339 (±8)
BSE-DoPrP^res	17/17	394 (±12)	13/13	274 (±9)[b]
Healthy cattle brain	0/12	>500[c]	ND	ND

ND, Not determined.

[a]One mouse was killed at 365 dpi and was IHC negative and showed no spongiform changes. This animal was not taken into account for incubation period calculations.

[b]One animal was found dead at 96 dpi and was PrP^res positive in the brain. This animal was taken out as an outlier because the cause of death could not be determined due to autolysis.

[c]An end point for negative controls was set at 506 dpi, data from other experiments show that healthy brain homogenate inoculated *tgBov* mice can survive >700 dpi (not shown).

BSE-DoPrP^res showed a similar pattern compared with the cattle BSE homogenate used as seed (Fig. 2). Once the species barriers were initially overcome *in vitro*, the derived BSE-RaPrP^res and BSE-DoPrP^res products were further amplified efficiently *in vitro* (data not shown). We selected saPMCA products from BSE seeded normal rabbit and dog brain homogenates and cattle brain derived BSE to be used as challenge inocula. All samples were subjected to at least 15 rounds of saPMCA (a 10^{-15} dilution of the initial seed which, according to its infectious titer, ensured no residual infectivity was left) after the first evidence of *in vitro* crossing of the species barrier.

Unaltered pathobiological BSE features after inoculation of rabbit, dog, and cattle adapted BSE prions in a bovine transgenic mouse model

To assess the infectivity of the newly *in vitro* generated prions, transgenic mice overexpressing bovine PrP^C (Castilla et al., 2003) were challenged intracerebrally. Brain homogenate from healthy, BSE-negative cattle was used as a negative control (Table 1).

A first passage through the transgenic mice of the cattle BSE and BSE-RaPrP^res or BSE-DoPrP^res inocula resulted in strikingly similar survival curves (Fig. 3) and no statistically significant differences were observed between the three inocula. The approximate difference in survival times of 40 d postinoculation (Table 1) might be a consequence of a low titer of the BSE-RaPrP^res and BSE-DoPrP^res samples amplified *in vitro* or because of a change of the prion strain after overcoming the cattle-rabbit and cattle-dog species barriers. A second passage through the transgenic mice was then performed to address this question. Upon this second passage, the incubation period of mice inoculated with cattle BSE was not statistically significantly different ($p = 0.9296$, Mann–Whitney test) to the first passage, whereas the other two inocula showed a significant reduction of the incubation periods compared with their respective first passages ($p = 0.006$ for BSE-RaPrP^res and $p = 0.000005$ for BSE-DoPrP^res, Mann–Whitney test). This could be interpreted as host adaptation process or as an increased titer compared with the *in vitro* generated inoculum. This reduction was especially greater for the BSE-DoPrP^res, which was significantly shorter compared with the incubation period of the second passage of cattle BSE inoculated mice ($p = 0.0003$, Mann–Whitney test). Shorter survival times than the original cattle BSE inocula have also been reported for sheep-passaged BSE (Espinosa et al., 2007). On the other hand, second passage BSE-RaPrP^res showed no statistically significant differences in the incubation period compared with cattle BSE ($p = 0.2207$, Mann–Whitney test).

Brain homogenates from the sick mice were obtained and the molecular weights and glycosylation patterns of PrP^res after PK digestion were studied by Western blotting. No differences were observed between the PrP^res of BSE infected cattle and the prions recovered from inoculating *tgBov* mice with either cattle BSE and rabbit or dog *in vitro* amplified prions. On second passage, the biochemical profile also remained unaltered (Fig. 4).

We then examined microscopically the brains of the inoculated mice. Consistent spongiform changes were observed in all BSE inoculated animals consisting of variably sized vacuoles in the neuropil of the gray matter (Fig. 5). The distribution of the lesion intensity throughout the brain was assessed semiquantitatively and showed that the three inocula yielded a very similar lesion profile on the first passage (data not shown). Lesions were particularly intense in the medulla oblongata, cerebellar nuclei, mesencephalon, thalamus, and striatum with significantly less involvement of the neocortex, cerebellar cortex, and piriform cortex. The hippocampal formation also showed some degree of spongy change. The brain lesion profiles of the second passage-inoculated animals remained unchanged compared with the first passage and also between the different inocula (Fig. 5); no significant differences were detected between the cattle BSE *tgBov* inoculated mice brain profiles and those of mice inoculated with BSE-DoPrP^res or BSE-RaPrP^res. However, the striatum (S) of the animals inoculated with the BSE-DoPrP^res showed significantly less spongiform lesions and PrP^d accumulation than the BSE-RabPrP^res or cattle BSE inoculated groups.

The immunolabeling patterns (monoclonal antibody 6H4) were consistent among the three differently challenged groups of mice and were mainly extracellular rounded plaque-like deposits of PrP^d. However, other patterns were observed also including fine punctuate to coarse granular, intraneuronal, perineuronal, and glial-associated (Fig. 5). The anatomical distribution of the PrP^d deposition intensity was also semiquantitatively assessed and the profile obtained was considerably similar to that of the lesions. On the second passage, the brains from BSE-DoPrP^res inoculated *tgBov* mice showed less PrP^d immunolabeling intensity in the cortices and hippocampus than mice from the other
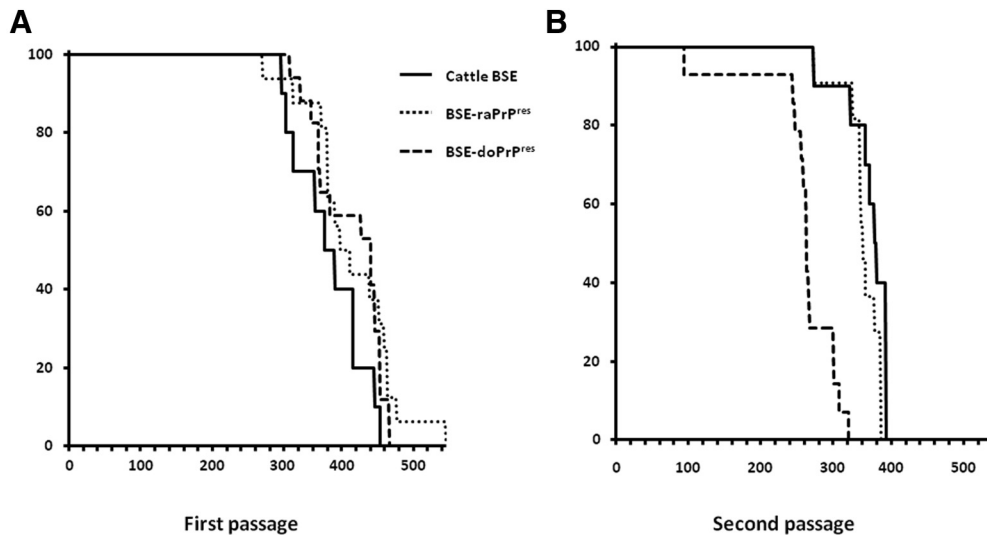
Figure 3. Survival Curves of cattle BSE, BSE-DoPrP^{res} and Ra-PrP^{res} inoculated *tgBov* mice. *A*, First passage; *B*, second passage. In the survival curves, the vertical axis represents the percentage of live animals and the horizontal axis the days postinoculation.

groups, which could be explained by the shorter incubation period (data not shown). Despite this difference in intensity, the profiles from all groups were similar in shape. The lesion profile showed a consistently lower PrP^d score in the striatum of BSE-DoPrP^{res} inoculated mice compared with the other two groups.

Preserved ability of rabbit adapted BSE prions to infect a human transgenic mouse model

Mice carrying the human *PRNP* gene were also inoculated intracerebrally with the *in vitro* generated BSE seeded products to assess their zoonotic potential. The cattle BSE inoculum did not result in disease in any of the animals (attack rate 0/10), a low infectivity rate that is consistent with published data on first passage of BSE in this model (Beringue et al., 2008; Padilla et al., 2011). Similarly, no animals inoculated with BSE-DoPrP^{res} succumbed to disease (0/6). However, 2 of 9 mice inoculated with BSE-RaPrP^{res} died at 560 and 699 dpi showing spongiform lesions and PrP^d deposits by IHC (Fig. 6; Table 2). The remaining animals died at different dpi without showing TSE-like signs, except for those associated to aging, and all were negative in the three tests performed to confirm TSE diagnosis (spongiform changes, IHC for PrP^d, and Western blot for PrP^{res}). Spongiform lesions were minimal in the TSE-positive transgenic mice and mixed with age-related spongiosis. PrP^d deposits were observed only in the thalamus and consisted of small foci of coarse granular PrP^d aggregates associated with the spongiosis. Overall, these results show that *in vitro* rabbit prions, likely enciphering a BSE strain, preserve the ability to infect humanized mice.

Several mice were found dead because of non-TSE-related causes but otherwise the animals were kept alive for their recognized lifespan (in the cattle BSE group up to 797 dpi, in the BSE-RaPrP^{res} group up to 704 dpi, and in the BSE-DoPrP^{res} group up to 853 dpi).

Discussion

Prion diseases have been known for a long time, especially scrapie in sheep and goats, and some diseases affecting humans, including Kuru and Creutzfeldt-Jakob disease. Yet the occurrence and identification of the BSE epizootic was a remarkable milestone in the history of prion disease as cattle had never before been affected by TSE and so were presumed resistant. The recognition

that BSE was zoonotic turned prion diseases of animals into a serious threat to public health, which resulted in an unprecedented crisis of confidence in consumers. Vast amounts of money were spent in numerous countries, not just to eliminate hundreds of thousands of infected animals, but also to establish radical changes related to disease control and surveillance, i.e., diagnostic tests, disposal of specified risk material, which could no longer be used in the food industry, etc. However, as prion diseases became the subject of profound scientific study, previously unknown prion strains have been discovered, many with unknown species susceptibilities and zoonotic potential. Thus, the susceptibility of different species to prions, particularly those that may come into close contact with humans, is a matter of continuous debate and study in the scientific community and great concern when determining health and safety policies.

Detailed study of naturally occurring prions is not enough to understand the behavior of these proteins in certain species. Before 1985, no one could have foreseen the massive cattle BSE epizootic. Hence, the scientific community is now particularly cautious when assessing the risk associated with host susceptibilities of various prions. Nevertheless, certain species display a limited or apparently null susceptibility to prion disease. Therefore, could one assume that such species would be resistant to all existing prion strains? If only one prion strain should be able to infect a new species, it might adapt to the new host and become easily transmissible. To address this we used all the tools available (i.e., *in vitro* amplification and transgenic mouse models) to evaluate the behavior of BSE prions in two historically considered prion disease resistant species: dogs and rabbits. BSE was chosen due to its zoonotic properties and because, as mentioned above, it is a promiscuous strain that has demonstrated infectivity in a wider range of species than most other prions. Even though these experimental conditions are far from modeling natural scenarios, they may enable us to predict the outcome of potential unforeseen species susceptibilities and epizootics as occurred with BSE (mad cow disease). During the last decade, *in vitro* replication studies endorsed PMCA as one of the most powerful technologies to overcome transmission barriers (Castilla et al., 2008; Green et al., 2008; Fernández-Borges and Castilla, 2010; Barria et al., 2011; Kurt et al., 2011; Yoshioka et al., 2011; Chianini et al., 2012).
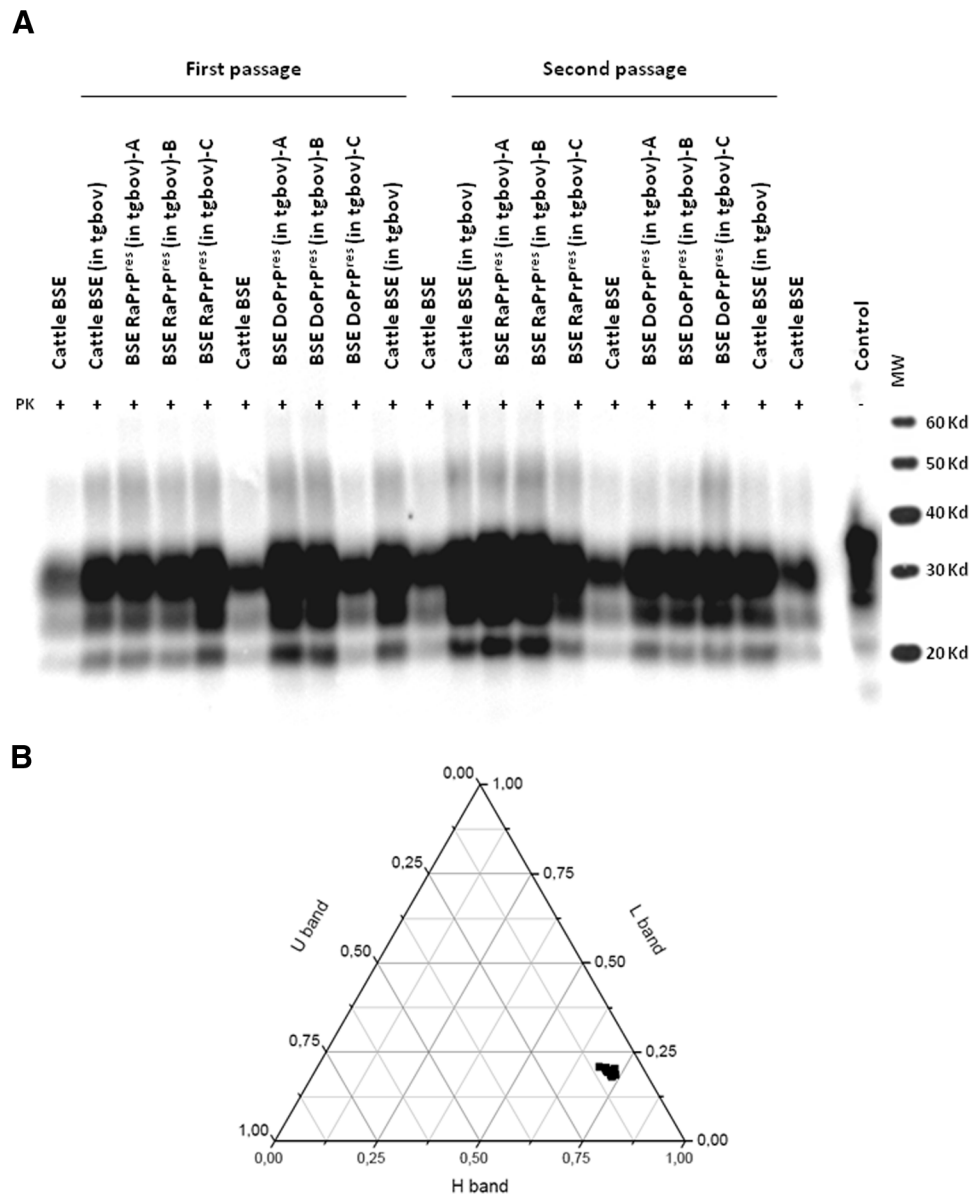
A

B

Figure 4. Comparative electrophoretic patterns. **A**, First and second passages of the different samples from *tgBov* mice (cattle BSE, BSE-DoPrP[res], and Ra-PrP[res]) were compared with natural cattle BSE. The glycoform ratios and the molecular weights of the PrP[res] are all indistinguishable from cattle BSE. All samples were digested with 40 μg/ml PK and analyzed by Western blot using monoclonal antibody D18. Cattle BSE and cattle BSE (in *tgBov*) were loaded several times in the gel for comparative purposes. Three different mice of each group (**A–C**) are represented for each type of sample and passage used. Control, Cattle brain homogenate. **B**, Triangular plot representing the glycoform proportions evaluated densitometrically from the above gel. Each axis represents the percentage of each band. H, Biglycosilated; L, monoglycosilated; U, unglycosilated. Notice that all wells show a remarkably similar band proportion.

Thus, dog and rabbit normal brain homogenates were seeded with BSE prions and submitted to *in vitro* amplification (saP-MCA) to try to overcome the species transmission barrier. Interestingly, rabbit PrP[C] could be misfolded with relative ease by any of the prion strains used TSE (Barlow and Rennie, 1976; Fernández-Borges et al., 2009; Chianini et al., 2012). This was rather surprising since this species had, until then, been considered resistant to TSE (Barlow and Rennie, 1976; Fernández-Borges et al., 2009; Chianini et al., 2012). However, dog brain homogenate could not be misfolded until the initial BSE prion seed concentration was raised considerably, and even with this modification only BSE was able to misfold dog PrP[C], which suggested a strong resistance of this species to BSE.

Comparison of the original cattle BSE prions with those generated by seeding rabbit and dog brain homogenate with BSE followed by *in vitro* saPMCA demonstrated that the biochemical strain properties of BSE were maintained in the new species, i.e., glycoform proportion and molecular weight after PK digestion. This suggests that the BSE prion's conformation is reliably transmitted to new PrP[C] species *in vitro*. However, to ensure that the BSE strain's pathobiological properties was also transmitted, particularly those regarding infectivity and the ability to cross certain species barriers, an *in vivo* approach was mandatory. The transgenic mouse model expressing the bovine *PRNP* gene was chosen as it had previously demonstrated to reproduce reliably BSE strain features. This model was key in determining that BSE acquired a different behavior regarding the length of the incubation period upon its passage through sheep, while maintaining other strain features, such as Western blot electrophoretical profile, molecular weight, and PK resistance (Espinosa et al., 2007). As
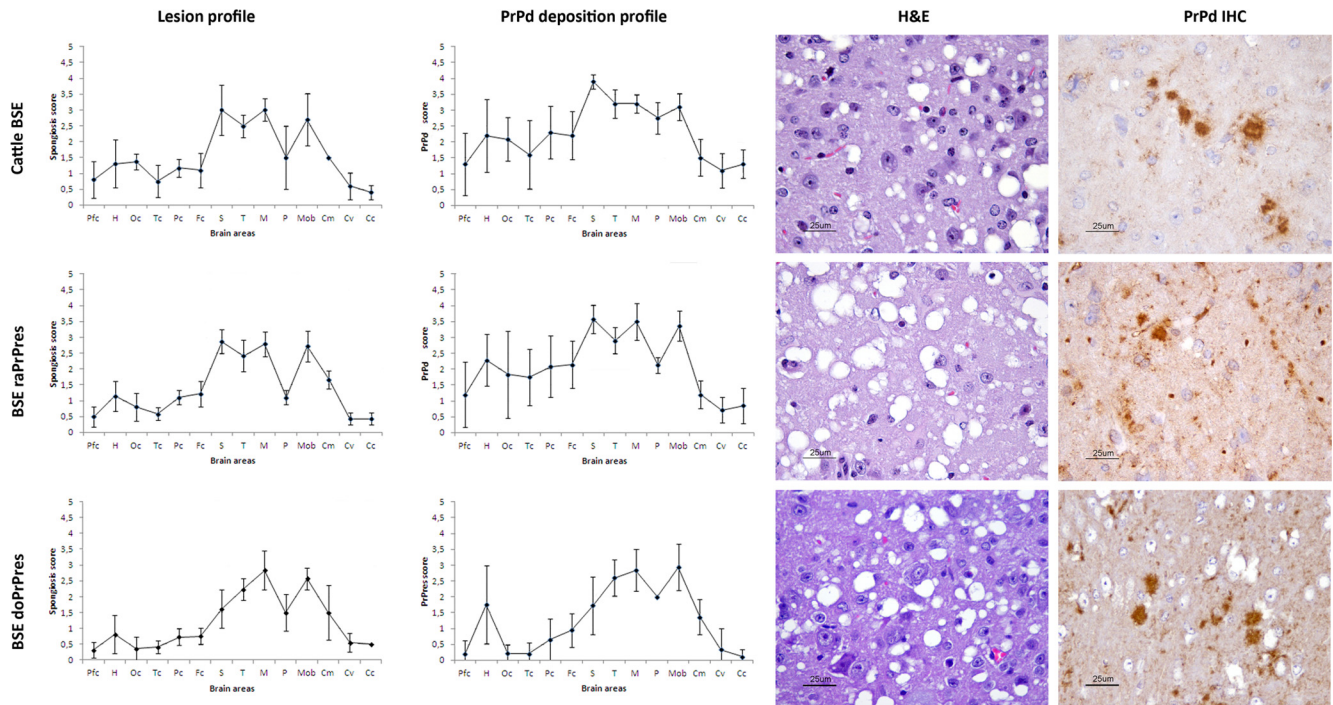
Figure 5. Histopathological characterization of the second passage of cattleBSE, BSE-DoPrP^res, and Ra-PrP^res in *tgBov* mice. Lesion profiles and PrP^d deposition profiles represent the mean semiquantitative scoring (0–4, vertical axis) of the spongiform lesions and the immunohistochemical labeling of PrP^d deposits, respectively, against 14 brain regions (horizontal axis: Pfc, piriform cortex; H, hippocampus; Oc, occipital cortex; Tc, temporal cortex; Pc, parietal cortex; Fc, frontal cortex; S, striatum; T, thalamus; M, mesencephalon; P, pons; Mobl, medulla oblongata; Cm, cerebellar nuclei; Cv, cerebellar vermis; Cc, cerebellar hemispheres). Vertical bars indicate the SD of the mean. H&E, Hematoxylin and eosin staining of the mesencephalon to evaluate the degree of spongiform change; PrP^d IHC, IHC for PrP^d deposits (brown pigment) in the mesencephalon (note the presence of abundant rounded, plake-like PrP^d deposits; 6H4 MAb 1:3000). Scale bars, 25 μm.
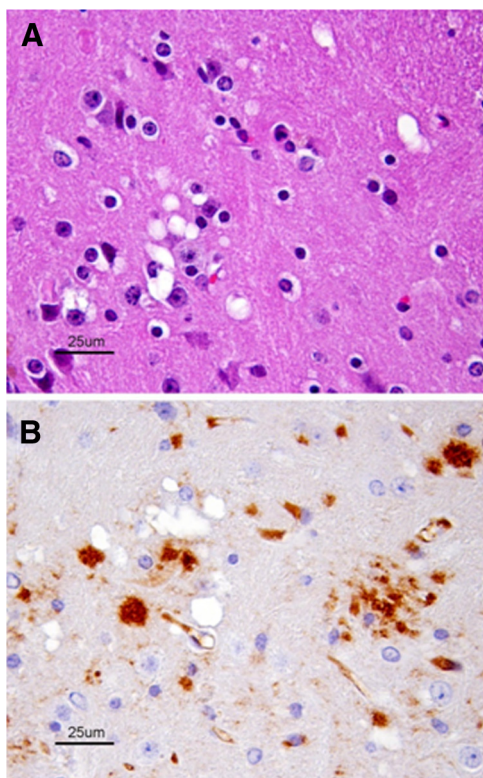

Figure 6. Characterization of BSE-RaPrP^res inoculated *tgHum* mice. *A*, Hematoxylin and eosin staining showing an area of spongiform change in the thalamus. *B*, PrP^d immunohistochemistry (MAb 6H4) of the same area depicts granular PrP^d deposits. Scale bars, 25 μm. Samples were digested with 40 μg/ml PK and analyzed using monoclonal antibody 6H4.

Table 2. Attack rates and survival times of inoculated *tgHum* mice

	First passage	
	Attack rate	Survival times (dpi)
Cattle BSE	0/9	203, 209, 234, 238, 247, 271, 468, 695, 797
BSE-RaPrP^res	2/9	170, 219, 306, 327, 371, 496, **560**, **699**, 705
BSE-DoPrP^res	0/6	271, 549, 677, 748, 818, 853
Healthy brain	0/6	370, 428, 463, 625, 692, 693

In bold, the two positive animals' survival time. The remaining data are survival times in dpi (i.e., days between inoculation and sacrifice).

predicted by the *in vitro* experiments (once initially converted, BSE-DoPrP^res and BSE-RaPrP^res easily amplified further dog and rabbit normal brain homogenates *in vitro*), both rabbit and dog adapted BSE efficiently infected *tgBov* mice with attack rates of 100%. Initially the incubation period was slightly longer for both inocula than that of cattle BSE, probably due to a lack of adaptation of the *in vitro* generated prions to the new host (bovine) prion. However, on second passage the incubation periods were significantly shortened. Interestingly the behavior of BSE-DoPrP^res was similar to that observed in sheep BSE where the biochemical, lesion, and immunohistochemical features were unchanged but with a reduction in the incubation period (Espinosa et al., 2007). The neuropathology and molecular signature of PrP^res found in *tgBov* mice showed no significant differences between cattle BSE and the two *in vitro*-generated prions, suggesting that the strain features had not changed upon passage through rabbit and dog brain homogenates.

The data shown here prove that the difficulties found in misfolding certain species of PrP^C molecules do not imply a loss of the pathobiological features encoded in the BSE structure. Compared with cat PrP^C (data not shown), dog PrP was tremendously

difficult to misfold *in vitro*. It could only be converted when a relatively large dose of BSE prion was used as seed. However, the similarity of dog PrP primary sequence, differing principally in just three residues (positions: 163, 181, and 189) with cat PrP (Lysek et al., 2005; Stewart et al., 2012), would predict a similar behavior to feline spongiform encephalopathy (FSE). In fact, FSE was one of the first nonbovine prions shown to maintain BSE strain features (Lezmi et al., 2006). Accordingly, once the bovine–canine barrier was surpassed, BSE pathobiological features remained stable in BSE-DoPrP^{res}.

However, the results obtained with BSE cannot be used to predict the behavior of other prion strains when adapted to these species *in vivo* or *in vitro*. Thus, instead of a species transmission barrier a strain transmission barrier should be considered as suggested by Scott and coworkers (Scott et al., 2005). Prion strains might be classified according to their ability to transmit to different species and also in relation to their adaptability to new hosts and their permissiveness to change. Therefore, BSE would be a strain with a low (or null) permissiveness to change and yet high adaptability to different environments. As such, the zoonotic behavior of BSE toward humans can be predicted regardless of the host infected with the BSE prion. Any species derivation of BSE, be it rabbit-BSE, dog-BSE, or any other version, is likely to encode a structure capable of misfolding human PrP^C. Second passage experiments in transgenic mice expressing human PrP (Tg340 mice) would have been useful to determine whether silent infection was present in mice inoculated with cattle BSE and BSE-DoPrP^{res} which seems to be a plausible assumption according to experiments published in this model (Padilla et al., 2011).

Our *in vitro* and *in vivo* results predict that, hypothetically, if BSE infected canids and leporids (considered, so far, prion resistant) it would maintain its pathobiological features; including zoonotic potential. This is particularly relevant with respect to rabbit amplified BSE as rabbits are eaten by humans. In conclusion, it is strongly recommended that no mammalian species be fed with animal protein potentially contaminated with BSE to prevent a new epizootic and zoonosis of unknown consequences.

Given the difficulties of performing infectivity studies in many natural hosts (without previous *in vitro* replication), transgenic mice have been generated expressing rabbit and dog *PRNP* gene which have been inoculated with cattle BSE, among other strains of interest, to test the *in vivo* susceptibility of these *PRNP* sequences. In addition, a detailed study of the primary amino acid sequences of bovine PrP compared with rabbit and dog PrP, focusing on the structural peculiarities of each amino acid change, is ongoing to explain the different PrP^C to PrP^d conversion abilities of each species.

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