Measuring prions causing bovine spongiform encephalopathy or chronic wasting disease by immunoassays and transgenic mice

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There is increasing concern over the extent to which bovine spongiform encephalopathy (BSE) prions have been transmitted to humans, as a result of the rising number of variant Creutzfeldt–Jakob disease (vCJD) cases. Toward preventing new transmissions, diagnostic tests for prions in livestock have been developed using the conformation-dependent immunoassay (CDI), which simultaneously measures specific antibody binding to denatured and native forms of the prion protein (PrP). We employed high-affinity recombinant antibody fragments (recFab) reacting with residues 95–105 of bovine (Bo) PrP for detection and another recFab that recognizes residues 132–156 for capture in the CDI. We report that the CDI is capable of measuring the disease-causing PrP isoform (PrP^{Sc}) in bovine brainstems with a sensitivity similar to that of end-point titrations in transgenic (Tg) mice expressing BoPrP. Prion titers were ~10^7 ID_{50} units per gram of bovine brainstem when measured in Tg(BoPrP) mice, a figure ~10 times greater than that determined by bioassay in cattle and ~10,000× greater than in wild-type mice. We also report substantial differences in $BoPrP^{Sc}$ levels in different areas of the obex region, where neuropathology has been consistently observed in cattle with BSE. The CDI was able to discriminate between PrP^{Sc} from BSE-infected cattle and Tg(BoPrP) mice as well as from chronic wasting disease (CWD)-infected deer and elk. Our findings argue that applying the CDI to livestock should considerably reduce human exposure to animal prions.

To apply the CDI for immunodetection of BSE and CWD prions, we generated a panel of recFabs binding to residues 90–115 of ungulate (hoofed mammals, including cattle, sheep, deer, and elk) PrP. Three IgG1κ antibody Fab libraries were displayed on the surface of filamentous phage and individually selected against a panel of PrP antigens, yielding >28 distinct recombinant antibody clones. The heavy-chain-complementary-determining region 3 (HCDR3) sequence of P Fab selected against recBoPrP(23–231) and chimeric mouse–bovine (MBo2M) PrP and displaying the most robust ELISA signals was: GAYYIKEDF.

Epitope mapping of O, P, and S Fabs demonstrated a single linear epitope lying between residues 96 and 105 of BoPrP. However, P Fab also displayed strong reactivity with PrP of other species (see Supplementary Fig. 1 online), which share the common epitope motif: HG(S,N)QWNKPSKPKTN. This epitope is present in human PrP as well as in all ungulate PrP sequences, including cattle, deer, elk, kudu, goat, and sheep. Using surface plasmon resonance to measure binding kinetics, we found that both the P and S antibody clones bound more tightly when expressed as chimeric human–mouse (HuM) proteins than when expressed as mouse Fabs (see Supplementary Table 1 online). Binding of the HuM-P Fab to BoPrP was particularly tight, with K_d values of 0.3 nM and 0.5 nM measured against MBo2MPrP(23–231) and BoPrP(90–145) antigens, respectively.

The CDI incorporating Eu-labeled HuM-P Fab was initially calibrated with recombinant MBo2M PrP that was refolded into a β-sheet conformation and designated rec β-MBo2M PrP (ref. 1). Rec β-MBo2M PrP was detected to a concentration as low as 20 ng/ml, and after sodium phosphotungstate (NaPTA) precipitation[2], to a concentration as low as 1 ng/ml, with <7% interassay variation (see Supplementary Fig. 2 online).

Using Eu-labeled HuM-P Fab in a manual CDI protocol[2], we determined the ratios of time-resolved fluorescence (TRF) signals recorded for native (N) and denatured (D) samples of normal bovine brain homogenate containing only normal, cellular PrP (PrP^C) to be = 2.37. In contrast, D/N ratios obtained for a BSE-infected brain homogenate containing a mixture of PrP^C and PrP^{Sc} were consistently >2.37. Similarly, the presence of $BoPrP^{Sc}$ could also be deduced if the difference between fluorescence signals measured in native and denatured aliquots (D–N) of the same sample[2] exceeded a calculated threshold value.

To evaluate the sensitivity of the CDI in detecting BSE and CWD prions, homogenates from pooled BSE-infected Tg(BoPrP)*Prnp*$^{0/0}$ mice or pooled CWD-infected deer brains were serially diluted into homogenates of normal mice or normal deer brain, respectively (Fig. 1A). These results demonstrate a robust quantitative response over a dynamic range of several orders of magnitude (Fig. 1A). The sensitivity of the CDI in detecting CWD prions was equal to or greater than that for the detection of BSE prions in Tg(BoPrP)*Prnp*$^{0/0}$ mice (Fig. 1A).

To determine the sensitivity of the automated CDI (aCDI) in detecting BSE prions, brainstem homogenates from BSE-infected cattle (Veterinary Laboratory Agency (VLA), New Haw, UK), were serially diluted into homogenates of normal bovine brain. We found a robust quantitative response and sensitivity limit at $10^{-3.7}$ dilution of BSE-infected brain (Fig. 1B). Introduction of the sandwich protocol using Fab D18 to capture BoPrP increased the sensitivity of the aCDI up to 20-fold (Fig. 1B).

Using a homogenate prepared from the medulla of a Hereford bull with BSE (case PG31/90), we did three separate titration series in parallel in Tg(BoPrP$^{+/+}$)4092/*Prnp*$^{0/0}$ mice, giving an average end-point titer of $10^{6.9}$ ID_{50} units/g of brain tissue (Fig. 1C). This finding compares with $10^{3.1}$ ID_{50} units/g of BSE-infected brain tissue titrated intracerebrally (i.c.) or intraperitoneally (i.p.) in RIII mice[3–6], and with 10^6 ID_{50} units/g reported for end-point titration in cattle[7]. These data indicate that Tg(BoPrP$^{+/+}$)4092/*Prnp*$^{0/0}$ mice are ~10 times more sensitive than cattle and >1,000 times more sensitive than RIII mice to infection with BSE prions. Any possibility that the BSE isolate from

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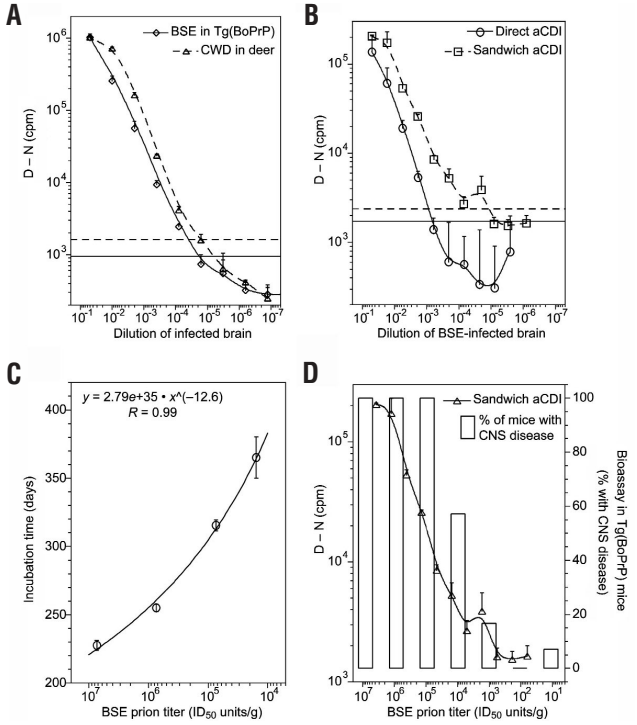

Figure 1. Similar sensitivity of the CDI and bioassays in Tg(BoPrP)*Prnp*$^{0/0}$ mice for BSE prions. (A) Dynamic range and analytical sensitivity of the manual direct CDI in detecting PrP^{Sc} in BSE- and CWD-infected brains. Brain homogenates from either pooled BSE-infected Tg(BoPrP)*Prnp*$^{0/0}$ mice or CWD-infected deer were serially diluted into homologous normal brain homogenate and tested by the CDI. The (D–N) value measured in counts per minute (c.p.m.) is directly proportional to the concentration of PrP^{Sc} (ref. 2). Data points and bars represent average ± s.d. obtained from three or four independent measurements. Manual CDI cutoff values were calculated by (mean + 3(s.d.)) using samples from uninoculated Tg(BoPrP) mice (solid horizontal line) and normal deer (broken horizontal line). (B) Direct and sandwich CDI protocols for the detection of $BoPrP^{Sc}$ were compared using pooled BSE-infected brainstem homogenates, serially diluted into normal brain homogenate prepared from the same brainstem area. The automated CDI was used for these studies. Cutoff values for the direct aCDI (solid horizontal line) and sandwich aCDI (broken horizontal line) were calculated by (mean + 3(s.d.)) and determined from 782 tests on 432 normal bovine brainstems. (C) Inverse exponential relationship between titers of BSE prions and incubation times in Tg(BoPrP$^{+/+}$)4092/*Prnp*$^{0/0}$ mice. The data points are the average ± s.e.m. calculated from three independent end-point titrations. (D) Direct relationship between $BoPrP^{Sc}$ detected by CDI and BSE prions measured in Tg(BoPrP)*Prnp*$^{0/0}$ mice. The (D–N) value is directly proportional to the concentration of PrP^{Sc} (ref. 2). The percentage of ill mice at each BSE sample dilution used in the bioassay was calculated from three independent end-point titrations.

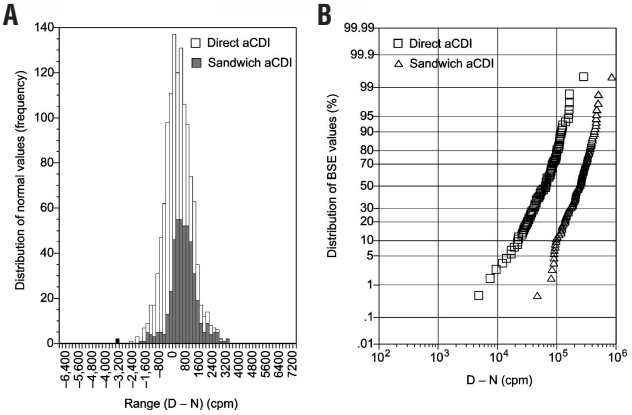

Figure 2. Statistical evaluations of the direct and sandwich automated CDI. Data for a group of (A) normal and (B) BSE-infected cattle. The obex area of each normal (n = 432) and BSE-infected brainstem (n = 100) was tested. Some samples were tested multiple times. Results are expressed as (D–N) differences in c.p.m.

PG31/90 contains an unusually high titer of prions seems highly remote, because previous comparison of PG31/90 with other BSE brain samples from the VLA gave similar or shorter incubation periods in Tg(BoPrP)4125/*Prnp*$^{0/0}$ mice[8].

The sensitivity of the aCDI applied to brain tissues of BSE-infected Tg(BoPrP)*Prnp*$^{0/0}$ mice approaches that of bioassay in cattle and Tg(BoPrP)*Prnp*$^{0/0}$ mice (Fig. 1A). Some variability in both CDI data and end-point titration experiments observed at high dilutions (from 10^{-4} to 10^{-6}) of prion-infected brain homogenates may be attributed to the stochastic distribution of prions in small aliquots used for bioassay (30 µl/mouse) and CDI (100 µl/well). One BSE infectious unit, which was calculated as the dilution point of BSE prions titrated with an average 50% survival rate in Tg(BoPrP)*Prnp*$^{0/0}$ mice, is equal to ~10^{-4} dilution of a 10% brain homogenate. Applying this, the 10^{-4} dilution that results in a 50% infection rate in the most sensitive bioassay in Tg mice correlates with a $10^{-4.6}$ BSE brain dilution that produces a positive result in 50% of aCDI tests (Fig. 1D). To examine the diagnostic performance of the aCDI, 100 confirmed BSE cases and 432 normal cows were tested. The distribution of (D–N) values in the normal group was Gaussian with a median value of –75 c.p.m. (Fig. 2A). A percentile plot of all measurements obtained from BSE-infected tissues expressed as the differences of (D–N) indicates a median value of ~66,000 c.p.m., and a ratio of ~880 for the median of positive to median of negative samples (Fig. 2B). Introduction of the sandwich aCDI increased the detection sensitivity for BSE prions ~5-fold for median values and up to 20-fold for samples with a low concentration of $BoPrP^{Sc}$ (Fig. 2). After 1,729 tests had been done, the aCDI identified all BSE cases with 100% accuracy, and no false positives occurred in the control group.

To understand the relatively broad distribution of (D–N) values within the BSE group, three BSE-infected brainstems were studied in greater detail. In each case, two adjacent, ~5-mm-thick cross sections were cut at the level of the obex. Transverse slices were cut from the midline to the periphery, yielding an average of 11 samples per brainstem (Fig. 3). Greatest (D–N) differences were seen in samples taken from the midline of the brainstem, but these values progressively decreased in samples collected more laterally (Fig. 3). Importantly, we found up to an eightfold variation in (D–N) values within one brainstem, indicating the importance of consistent sampling for accurate diagnostic performance.

Previously, we plotted the D/N ratio against the PrP^{Sc} concentration to distinguish different prion strains passaged in hamsters[2]. Because the PrP^{Sc} concentration is directly proportional to (D–N) value, we plotted the D/N ratio against the (D–N) difference (Fig. 4). The D/N ratio depends on the antibody binding affinity to PrP^{Sc} conformers, in which D is considered a reference point and N depends on the antibody binding affinity and the epitope availability in undenatured PrP^{Sc}. Thus, the ratio gives simple quantitative information about the native conformation of PrP^{Sc}, which is prion strain-specific[2,9]. Using this approach, we found that three individual cases of BSE- or vCJD-infected Tg(BoPrP) mice showed D/N ratios within the range of BSE-infected cattle, indicating similar conformational characteristics with a somewhat higher concentration of $BoPrP^{Sc}$ accumulating in the brains of Tg mice.

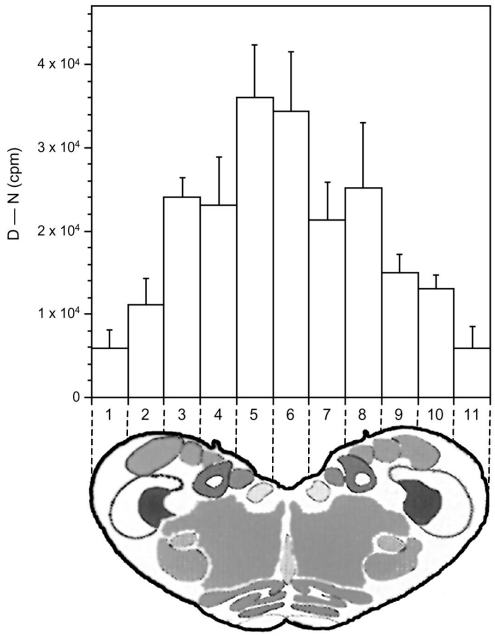

Figure 3. Transverse distribution of PrP^{Sc} in bovine brainstems at the level of the obex as determined by CDI analysis correlates with known BSE pathology. The averaged distribution of (D–N) values measured by CDI using six slices from three brainstems taken from BSE-infected cattle is shown in the upper panel. The numbered position of each slice corresponds to the anatomical location and structures in the lower panel drawing. Values (average ± s.e.m., $n = 6$) are directly proportional to the concentration of PrP^{Sc} (ref. 2). The data in the lower panel diagram were compiled from histoblots[20] and standard pathological techniques[21]; the light-to-dark shading indicates, from mild to intense, respectively, the severity of vacuolation and degree of PrP^{Sc} staining.

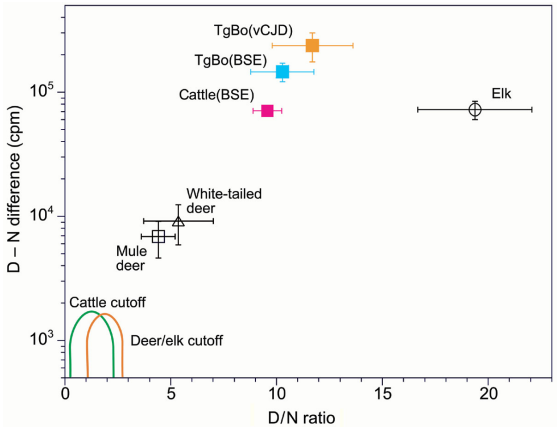

Figure 4. Different conformational characteristics of ungulate prion strains revealed by direct CDI. CDI data were plotted as D/N ratios against (D–N) values recorded for BSE-infected British cattle ($n = 100$; magenta square), first passage of pooled brain homogenates from BSE- and vCJD-infected Tg(BoPrP)$Prnp^{0/0}$ mice (cyan square and orange square, respectively), CWD-infected mule deer ($n = 10$; open square), white-tailed deer ($n = 6$; open triangle), and elk ($n = 19$; open circle). The arcs link cutoff values for differences and ratios in normal US cattle (green; $n = 432$) and in normal deer and elk (orange; $n = 60$).

We applied the same plot analysis with CWD samples. However, CWD may not be compared directly with BSE because of the G→S polymorphism at position 97 within the P Fab epitope. When comparing results from different CWD hosts sharing the same sequence, samples from white-tailed and mule deer grouped in the same area, whereas samples from elk grouped separately (Fig. 4). This finding suggests a different PrP^{Sc} conformation in elk from that in white-tailed and mule deer. Whether this observation indicates prion strain differences between elk and deer remains to be established.

For years, detection of protease-resistant (r) PrP^{Sc} by immunoblot or immunohistochemistry represented the only means of identifying prions in tissue *in vitro*. Reliance on the complete enzymatic hydrolysis of ubiquitous, immunoreactive PrP^{C}, however, has limited both the specificity and sensitivity of these assays[6,10,11]. In our studies, recFabs recognizing ungulate PrP with high affinity and specificity have been incorporated into a high-throughput aCDI, creating a rapid and sensitive methodology for detecting BSE and CWD prions. The CDI, directly identifying PrP^{Sc} through its distinct conformation, may be conducted independently of proteolytic treatments. Consequently, the CDI can detect potentially large *in vivo* concentrations of protease-sensitive (s) PrP^{Sc} molecules[2]. In hamster brains, $sPrP^{Sc}$ is found much earlier than $rPrP^{Sc}$ (unpublished data); whether a similar situation occurs in cattle or other ungulates remains to be determined.

The performance characteristics and advantages of the aCDI for detecting PrP^{Sc} in BSE- and CWD-infected brainstems include (i) detection of both $sPrP^{Sc}$ and $rPrP^{Sc}$; (ii) a readily scalable, quantitative diagnostic system using robotic protocols to maintain specificity and sensitivity; (iii) automated quality control and data processing; (iv) extremely accurate diagnostic for detection of PrP^{Sc} in brainstems collected postmortem; (v) prion positive:control ratio as high as 1,000 for BSE- and 4,000 for CWD-infected samples; (vi) interassay variation <7%; (vii) detection limit for BSE prions similar to bioassays in Tg(BoPrP)$Prnp^{0/0}$ mice; and (viii) potential for rapid prion strain typing.

To insure public safety, prion assays should detect one infectious unit. However, prion titers can be greatly altered by the host in which measurements are made. For example, BSE infectivity measured in cattle showed that earlier studies using an RIII mouse bioassay[3] underestimated BSE prion titers by a factor of ~1,000 (refs. 5, 7). We report here that Tg(BoPrP)$Prnp^{0/0}$ mice, expressing multiple copies of the bovine *PRNP* gene, are ~10 times more sensitive to infection with BSE prions than cattle. This finding indicates that previous attempts to quantify BSE and scrapie prions in milk or nonneural tissues, such as muscle, may have underestimated infectious titers by up to a factor of 10^4, raising the possibility that prions could be present in these products in sufficient quantities to pose some risk to humans[12]. Additionally, in the studies reported here, we found that PrP^{Sc} levels can vary by site within a brainstem (Fig. 3) by a factor of 8. This suggests that multiple samples from each animal are required to minimize the number of false negatives.

We demonstrate that the CDI is capable of measuring PrP^{Sc} in bovine brainstems with a sensitivity similar to the infectivity levels determined by end-point titrations in Tg(BoPrP) mice (Fig. 1). Although the brain is known to contain the highest levels of PrP^{Sc} in all prion diseases, the inaccessibility of this tissue will continue to hinder epidemiologic investigations of these diseases. It will be important to apply the CDI to the development of an antemortem test for prions. Using the CDI, we have detected $sPrP^{Sc}$ consistently in the blood of rodents infected with prions[13]. Using western blots and ELISAs, we have detected $rPrP^{Sc}$ in the hindlimbs of mice[12]. Whether blood or muscle will be a suitable matrix for the development of an antemortem test for prions remains to be determined.

Experimental protocol

Immunization and recovery of BoPrP-specific recFabs. Six-week-old *Prnp*-ablated (*Prnp*$^{0/0}$) mice were injected i.p. with 100 μg of a synthetic BoPrP(96–115) peptide crosslinked to keyhole limpet hemocyanin and fully emulsified in RIBI adjuvant. Three mice with the most robust and specific anti-PrP serum IgG titers received a final boost with 25 μg of unconjugated BoPrP(96–115) peptide and were sacrificed six days later. Three IgG Fab libraries displayed on the surface of filamentous phage were independently prepared from spleen and bone marrow RNA derived from each of these animals[14].

Phage antibody libraries were individually panned against a panel of different recombinant and synthetic PrP antigens immobilized onto ELISA wells[14]. The recovery and epitope recognition characteristics of Fab D18 were described[15].

Expression and purification of HuM Fabs. Fabs P, S, and D18 were expressed in bacteria as both mouse and HuM Fabs, in which the murine heavy- and light-chain variable genes were fused to genes encoding human CHγ1 and C_K sequences[16]. The HuM recFabs were expressed and fermented in *Escherichia coli* 33B6 competent cells and purified as described[16]. Selected antibody clones were also inserted sequentially into a eukaryotic expression vector containing human IgG1 constant region and human κ constant region genes and expressed in Chinese hamster ovary (CHO) cells[17].

Surface plasmon resonance. Recombinant BoPrP(23–231), SHaPrP(29–231), or synthetic antigens were immobilized to carboxymethyl groups present on the surface of CM5 sensor chips using *N*-hydroxysuccinimide and *N*-ethyl-*N*'-((dimethylamino)propyl)-carbodiimidehydrochloride[18]. Kinetic constants for the binding of PrP-specific recFabs or IgG for a series of concentrations were analyzed globally using BiaEval 3.1 software (Biacore).

Preparation of brain homogenates. Slices from the obex area of the brainstem were homogenized to a final 15% (wt/vol) in 4% (wt/vol) Sarkosyl in PBS, pH 7.4. The homogenate was diluted to 5% (wt/vol) using PBS containing 5 μg/ml of proteinase K and incubated for 60 min at 37°C. After clarification, samples were precipitated with 0.32% NaPTA. After a 60 min incubation at 37°C, samples were centrifuged at 14,000 *g* in a Jouan MR23i centrifuge for 15–30 min, and resuspended pellets were assayed by the CDI.

Direct and sandwich CDI for ungulate PrP^{Sc}. The principle, development, calibration, and calculation of PrP^{Sc} concentration from CDI data have been described[2]. The CDI data described in this paper were generated with recFab HuM-P labeled with Eu-chelate. Each sample was divided into two aliquots: (i) untreated (designated native) and (ii) mixed to a final concentration of 4 M GdnHCl and heated for 5 min at 80°C (designated denatured). Both samples were diluted with H_2O containing protease inhibitors, and aliquots were loaded on a 96-well black polystyrene plate (Packard, Meriden, CT) that had been activated with 0.2% glutaraldehyde for direct CDI or coated overnight with 5 μg/ml recFab D18 in sodium bicarbonate buffer, pH 8.6 for sandwich CDI. The plates were incubated for 2 h and then blocked with Tris-buffered saline (TBS), pH 7.8, containing 0.5% BSA (wt/vol) and 6% sorbitol (wt/vol) for 1 h at room temperature. The plates were washed with TBS containing 0.05% (vol/vol) Tween-20, incubated with Eu-labeled recFab HuM-P for 2 h, washed, then developed in enhancement solution (Wallac Inc., Turku, Finland). Signals were counted on a Discovery dual-wavelength, time-resolved fluorometer (Packard).

aCDI with sample tracking and data processing. The scaled-up aCDI protocol was developed for the Genesis Robotic Sample Processor 150 liquid-handling station (Tecan, Research Triangle Park, NC). All data were analyzed using a custom-built MS SQL interface to distinguish between positive and negative samples by comparing with internal standards and threshold values.

End-point titration of BSE prions in Tg(BoPrP)*Prnp*$^{0/0}$ mice. We established Tg(BoPrP$^{+/+}$)4092/*Prnp*$^{0/0}$ mice that are homozygous for the bovine transgene array. The inoculum was a 10% (wt/vol) homogenate prepared from the medulla of a Hereford bull with BSE (case PG31/90). Three separate dilution series were done in parallel, and 30 μl from each dilution were inoculated i.c. into groups of five mice. Triplicate end-point titrations of BSE prions were done; titers were calculated according to the methods of Karber as well as Reed and Munch[19], and the average from these three titrations was determined. The results are expressed as the median infective dose (ID_{50}) per gram of tissue.

Note: Supplementary information is available on the Nature Biotechnology website.

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Competing interests statement

The authors declare competing financial interests: see the Nature Biotechnology website (http://biotech.nature.com) for details.

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