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High Titers of Transmissible Spongiform Encephalopathy Infectivity Associated with Extremely Low Levels of PrP^{Sc} *in Vivo*[*] [S]

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Diagnosis of transmissible spongiform encephalopathy (TSE) disease in humans and ruminants relies on the detection in post-mortem brain tissue of the protease-resistant form of the host glycoprotein PrP. The presence of this abnormal isoform (PrP^{Sc}) in tissues is taken as indicative of the presence of TSE infectivity. Here we demonstrate conclusively that high titers of TSE infectivity can be present in brain tissue of animals that show clinical and vacuolar signs of TSE disease but contain low or undetectable levels of PrP^{Sc}. This work questions the correlation between PrP^{Sc} level and the titer of infectivity and shows that tissues containing little or no proteinase K-resistant PrP can be infectious and harbor high titers of TSE infectivity. Reliance on protease-resistant PrP^{Sc} as a sole measure of infectivity may therefore in some instances significantly underestimate biological properties of diagnostic samples, thereby undermining efforts to contain and eradicate TSEs.

The transmissible spongiform encephalopathy (TSE)[4] diseases (also known as prion diseases) are infectious, fatal neurodegenerative diseases of animals, which include Creutzfeldt-Jacob disease (CJD) in humans and bovine spongiform encephalopathy (BSE) in cattle. The true identity of the infectious agent responsible for these diseases is not known. However, it has been proposed that TSE disease is caused by an abnormal form of the host glycoprotein, PrP (1). The abnormal, disease-associated form of the protein (PrP^{Sc}), is partially protease-resistant and detergent-insoluble unlike the normal cellular conformer (PrP^{C}), and is seen to accumulate in diseased tissues. The prion hypothesis predicts that PrP^{Sc} alone is the infectious agent of TSE and is able to induce the conversion of endogenous PrP^{C} into the abnormal form during disease (2).

Most human TSE diseases are familial or sporadic, but disease can also be acquired by surgical intervention (3) or blood transfusion from infected individuals (4–9), or possibly from the consumption of BSE-infected meat products, the presumed cause of variant CJD (vCJD) (10). The extent to which vCJD infection in particular is present in the United Kingdom population is unknown, but recent research has suggested there may be a higher rate of subclinical or preclinical vCJD than previously thought in different human PrP genotypes (7, 11–13). Although BSE is declining in the United Kingdom, cases have now been observed in cattle in countries that have not previously reported BSE. It is also unknown whether the agent responsible for BSE has re-entered the human food chain following transmission to sheep. For these reasons a high level of active and passive surveillance of ruminants is required at slaughter to monitor and prevent TSE-infected material from entering the human food chain. The introduction of ante-mortem surveillance in the human population is also critical to prevent the human-to-human transmission of vCJD by blood transfusion or surgical procedures. This will be of particular importance if subclinical disease proves to be a significant risk in vCJD transmission (12, 13).

Positive identification of TSE infectivity can only be demonstrated conclusively by transmission of disease to laboratory animals. Such assays are time-consuming, due to long incubation times, and expensive, and are therefore not suitable for the rapid diagnosis of all ante- or post-mortem samples. Current diagnostic tests instead rely on the detection of disease-associated PrP^{Sc} in samples taken from brain post-mortem. The development of ante-mortem diagnostic tests is also being based around more sensitive assays for PrP^{Sc}. Several diagnostic tests are available commercially, and most require proteinase K (PK) treatment of tissue homogenates to isolate disease-specific PK-resistant PrP^{Sc} (PrP-res). It has not yet been definitively proven that PrP^{Sc} is the TSE infectious agent, and whether it is present in all infected tissues. Studies using 263K hamster scrapie have shown a strong correlation between PrP-

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[4] The abbreviations used are: TSE, transmissible spongiform encephalopathy; CJD, Creutzfeldt-Jacob disease; vCJD, variant Creutzfeldt-Jacob disease; PK, proteinase K; GSS, Gerstmann Sträussler Scheinker; CDI, conformation-dependent immunoassay; IP, immunoprecipitation; IHC, immunohistochemistry; mAb, monoclonal antibody; BSE, bovine spongiform encephalopathy; PrP-res, PK-resistant PrP^{Sc}; $sPrP^{Sc}$, PK-sensitive form of PrP^{Sc}; ELISA, enzyme-linked immunosorbent assay; d/n, ratio of denatured to native signal; Wt, wild-type.

res and infectivity (2, 14, 15). However, other studies have demonstrated the transmission of disease from infected animals that appear to lack significant levels of PrP-res (16–19). In such cases it has been suggested that a PK-sensitive form of PrP^{Sc} ($sPrP^{Sc}$) may represent the infectious agent (20–22). Hence it is possible that infectivity may be associated with a specific isoform of abnormal PrP. The identification of this specific conformer is imperative for the future of TSE diagnosis. If present, large amounts of PrP^{Sc} may be a clear indication of the presence of infectivity in a tissue sample. However, if TSE infectivity does not always associate with high levels of PrP^{Sc}, current diagnostic methods may fail to identify all animals with TSE disease and may not provide a realistic estimate of the level of infectivity in an infected tissue. For the purposes of this study, PrP^{Sc} is used to define all abnormal forms of PrP, whereas PrP-res specifically defines PK-resistant PrP, and $sPrP^{Sc}$ defines PK-sensitive forms of PrP^{Sc}.

We have previously identified two mouse models of TSE disease (18, 19) that indicate that the association between PrP-res and infectivity is not as straightforward as predicted by the prion hypothesis. Unlike wild-type controls, transgenic mice homozygous for a targeted mutation at amino acid 101 (proline to leucine) in endogenous murine PrP (101LL) develop clinical TSE disease following inoculation with hamster 263K scrapie or human Gerstmann Sträussler Scheinker (GSS) P102L disease (patient shown to contain vacuolar pathology and PrP-res at post-mortem) (18, 19). Pathological analysis of brain tissue from these mice (101LL/GSS and 101LL/263K) showed TSE-associated vacuolization, and the disease could be further transmitted to 101LL mice with short incubation times of 100–160 days (18, 19). Such incubation times were indicative of a high titer of infectivity in the 101LL/GSS and 101LL/263K tissues, yet analysis by immunoblot revealed that most animals contained extremely low levels of PrP-res, and several contained no detectable PrP-res at all (18, 19). However, the presence of high titers of infectivity cannot be proven by a short disease incubation time. To establish the true relationship between PrP^{Sc} and infectivity we have now performed detailed and quantitative analyses of the disease in these mice. The ID_{50} (dilution at which 50% of the animals become infected) and titer of infectivity in several 101LL/GSS- and 101LL/263K-infected brains have been established by bioassay. Corresponding levels of PrP-res in the same tissues have also been established semi-quantitatively by immunoblot. These analyses have shown no relationship between infectivity titer and PrP-res level. Moreover no other disease-associated forms of PrP were detectable in these tissues. Thus within our model system there is a clear dissociation between titer of infectivity and level of PrP^{Sc}.

EXPERIMENTAL PROCEDURES

Transgenic Mouse Lines and Tissues—Inbred gene-targeted transgenic mouse line 101LL and the corresponding inbred 129/Ola wild-type control line have been described previously (18). 101LL/GSS tissues were produced by inoculation of 101LL transgenic mice with 1% brain homogenate prepared from the occipital cortex of a GSS P102L brain showing numerous multicentric plaques and abundant PrP-res by immunoblot. The individual was methionine 129 homozygous with a confirmed proline to leucine mutation at codon 102.[5] 101LL/263K tissues were produced by inoculation of 101LL transgenic mice with 1% brain homogenate from a 263K-infected hamster. Control tissues were produced by ME7 inoculation of 129/Ola wild-type mice and 101LL transgenic mice.

Preparation of Inocula—Separate inocula were prepared from the brains of two 101LL/GSS- and three 101LL/263K-infected mice with terminal TSE disease, which had been shown by immunohistochemical (IHC) analysis to contain extremely low levels of PrP deposition. Inocula were also prepared from brains of one wild-type and one 101LL mouse with terminal ME7 scrapie as controls. A 10% homogenate of each sample was prepared in sterile saline prior to use as an inoculum. This inoculum was then used to produce a series of 10-fold dilutions from 10^{-2} to 10^{-9} in sterile saline. Each dilution (20 μl) was inoculated intracerebrally under anesthesia into groups of 101LL mice for 101LL/ME7, 101LL/GSS, and 101LL/263K tissues, or wild-type 129/Ola mice for Wt/ME7 tissue. All experimental protocols were submitted to the Local Ethical Review Committee for approval before mice were inoculated. All experiments were performed under license and in accordance with the UK Home Office Regulations (Animals (Scientific Procedures) Act of 1986).

Scoring of Clinical TSE Disease—The presence of clinical TSE disease was assessed as described previously (23). Animals were scored for clinical disease without reference to the genotype of the mouse. Genotypes were confirmed for each animal by PCR analysis of tail DNA at the end of the experiment. Incubation times were calculated as the interval between inoculation and cull due to terminal TSE disease. Mice were killed by cervical dislocation at the terminal stage of disease, at termination of the experiment (between 500–700 days), or for welfare reasons due to intercurrent illness. The proportion of mice showing positive vacuolar pathology was calculated for each group, and the ID_{50} (dilution at which 50% of the mice became infected) was determined using the Karber method (24). This value was used to calculate the number of infectious units per gram wet weight of tissue (IU/g).

Genotyping of Mouse Tail DNA—A 2- to 3-cm portion of tail was removed post-mortem from each mouse. DNA was prepared, and the PrP genotype of each mouse was confirmed as described previously (18).

Immunoblot Analysis and Quantification of PrP-res—For immunoblot analysis, residual inocula (10% saline homogenate) were mixed with an equal volume of 2× Nonidet P-40 buffer (2% Nonidet P-40, 1% sodium deoxycholate, 300 mM NaCl, 100 mM Tris/HCl, pH 7.5) and further homogenized in a microcentrifuge tube using 20–30 strokes with a pre-cooled centrifuge tube pestle (Anachem). The homogenate was centrifuged at 11,000 × *g* for 10 min at 10 °C to remove cellular debris, and the supernatant stored in 50-μl aliquots at −70 °C. For quantification of PrP-res levels in each tissue, homogenates were digested with 20 μg/ml PK at 37 °C for 1 h. Digested homogenates were diluted to 1%, and 2-fold serial dilutions were prepared using PK-treated normal brain homogenate as

[5] J. W. Ironside and M. W. Head, personal communication.

the diluent to keep overall protein concentrations constant. Diluted samples were mixed with sample loading buffer and sample reducing agent (Invitrogen) and loaded across two 12% Tris/glycine polyacrylamide gels (Invitrogen) at concentrations ranging from 1 mg/ml to 3.9 μg/ml (200 μg to 0.8 μg of wet weight tissue equivalent). 50 ng of recombinant PrP was loaded onto each gel as an internal control. After separation, proteins were transferred onto a polyvinylidene difluoride membrane by electroblotting, and PrP was detected with mAb 8H4 (West Dura ECL substrate, Pierce). Monoclonal antibody 7A12 and polyclonal antibody 1B3 were also used to confirm the low PrP-res levels in 101LL/GSS and 101LL/263K tissues. Images were captured on both x-ray film and by a Kodak Digital Image Station 440. Experiments were repeated in duplicate or triplicate depending on sample availability.

Digital images of each gel were analyzed using Kodak ID software, and PrP-res levels were expressed as pixel intensities. Samples were normalized across the two blots and quantified using the recombinant PrP controls as standards. Each value was multiplied by the dilution factor, and an average was taken for all samples run per tissue to determine the level of PrP-res per gram wet weight brain tissue in each model. This value, combined with the titer of TSE infectivity measured in each tissue (IU/g) was used to calculate the number of molecules of PrP-res per infectious unit for each tissue as in Equations 1–3.

$$\text{Number of PrP-res molecules per g of tissue} = n \quad \text{(Eq. 1)}$$

$$n = [\text{PrP-res per g/Avagadro's number } (6.02 \times 10^{23})]/\text{molecular weight PrP } (30{,}000) \quad \text{(Eq. 2)}$$

$$\text{Number of molecules PrP-res per infectious unit} = n/\text{titer (IU/g)} \quad \text{(Eq. 3)}$$

Measurement of Alternative Forms of PrP—The PK resistance of PrP in all samples was analyzed by digestion with a range of PK concentrations. Individual 9-μl aliquots of each 5% Nonidet P-40 brain homogenate were incubated at 37 °C for 1 h with PK concentrations ranging from 1 to 20 μg/ml. The reaction was terminated by addition of phenylmethylsulfonyl fluoride to 1 mM, and samples were analyzed by SDS-PAGE and immunoblotting as described above.

For "cold PK" digestion, samples (10% homogenate) were incubated with 250 μg/ml PK on ice for 1 h. Digestion was stopped by the addition of phenylmethylsulfonyl fluoride to 1 mM. Samples were de-glycosylated with peptide *N*-glycosidase F (New England Biolabs) following the manufacturer's instructions and analyzed by SDS-PAGE and immunoblotting.

CDI Analysis—Samples were analyzed for the presence of PrP^{Sc} using conformation-dependent immunoassay (CDI) as described by Safar *et al.* (20). Briefly, abnormal PrP was precipitated from brain homogenates of 101LL/GSS, 101LL/263K, and 101LL/ME7 infected mice and uninfected 101LL mice using sodium phosphotungstate, and pellets were resuspended in either distilled water or 4 M guanidine hydrochloride to produce native and denatured samples. 4 M guanidine hydrochloride samples were further heat-denatured at 80 °C for 6 min. Samples were added to 96-well plates coated with mAb FH11, and PrP levels were detected using europium-labeled mAb 7A12 and a Victor 2 ELISA plate reader (PerkinElmer Life Sciences). The ratio of denatured to native signal (d/n) was calculated for each tissue to determine the presence of PrP^{Sc}.

Immunoprecipitation of PrP^{Sc}—Laterally bisected brain halves from 101LL transgenic mice were homogenized at 10% (w/v) in Tris-buffered saline and diluted to reach a concentration of 5% (w/v) in Tris-buffered saline containing 1% Triton. Homogenates were sonicated for three pulses of 4 s and clarified by centrifugation at 400 × *g* for 10 min at 4 °C. Phenylmethylsulfonyl fluoride was added to all samples to a concentration of 2 mM. Each sample was analyzed by dot blot to estimate the total PrP content. Briefly, brain homogenates were serially diluted (1:1) in Tris-buffered saline containing 1% Triton then denatured in Tris-SDS sample buffer at 100 °C for 5 min. Equivalent amounts of each sample were then deposited on a nitrocellulose membrane and left until dry. The membrane was probed with mAb 6H4 (Prionics) and a horseradish peroxidase-labeled anti-mouse secondary antibody (Pierce). The resulting signals were compared semi-quantitatively. These data were used to ensure equal PrP input into each individual immunoprecipitation (IP) reaction. For each IP reaction, the motif grafted antibodies or control antibodies were incubated at 10 μg/ml final concentration for 2 h at room temperature in a reaction mixture with 1% Triton. Rabbit anti-human antibodies (Jackson) coupled to magnetic Dynabeads (Dynal) were used to capture the PrP-specific antibodies as described (25, 26). Immunoblot membranes were probed with mAb 6H4 and developed using the ECL femtomolar kit (Pierce).

RESULTS

101LL Mice Infected with 263K and GSS P102L Show Little PrP Deposition in Brain—Brain tissue from 101LL transgenic mice, which showed TSE clinical signs and TSE-associated vacuolar pathology following inoculation with hamster 263K scrapie or human GSS P102L (18, 19), was screened for PrP deposition by IHC using anti-PrP mAb 6H4. As previously demonstrated, 101LL/GSS- and 101LL/263K-infected mice had low levels of PrP deposition in the brain, despite having confirmed TSE disease. Three 101LL/263K- and two 101LL/GSS-infected tissues, which showed extremely low PrP deposition in the brain, were selected for further analysis by bioassay (Fig. 1 and Table 1). In each case, PrP deposition was restricted to the thalamus and, in most cases, was only visible as small grainy deposits under high power microscopy (Fig. 1, *F–H*). Low or undetectable levels of PrP-res in each brain homogenate were confirmed by immunoblot following PK treatment of residual inoculum (Fig. 2).

High Levels of Infectivity Can Be Measured by Bioassay of 101LL/GSS and 101LL/263K Brain Tissue—Although short incubation times in mice can be indicative of high levels of TSE infectivity in an inoculum, the actual level can only be determined by establishing the ID_{50} (dilution at which 50% of the animals become infected) for the inoculum. Infectivity titers were therefore established for the five selected tissues: 101LL/263K(a), 101LL/263K(b), 101LL/263K(c), 101LL/GSS(d), and 101LL/GSS(e) (Table 1). It was considered extremely important in these experiments that, as far as possible, a single brain be

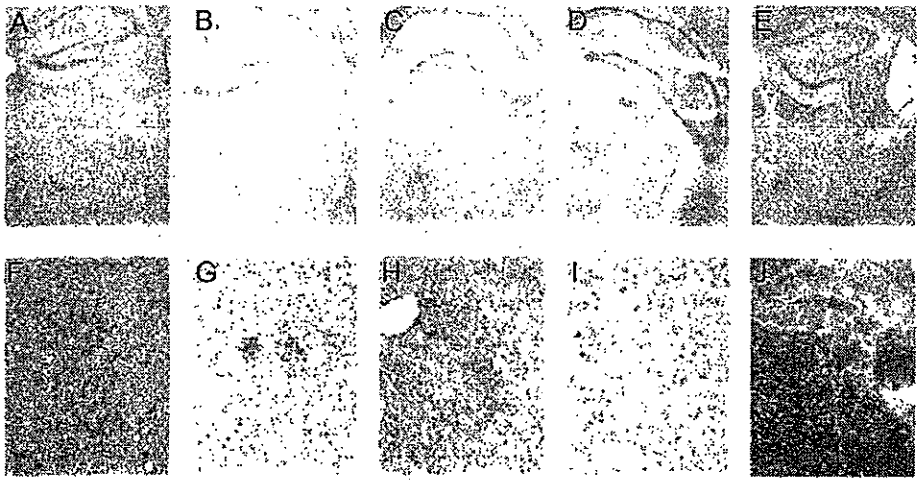

FIGURE 1. **Low levels of PrP deposition in 101LL/GSS- and 101LL/263K-infected brain.** Immunohistochemistry was performed on sections of brain from 101LL/263K- and 101LL/GSS-infected mice using mAb 6H4 to determine the levels of PrP deposition. ME7-infected control mouse brain was stained as control (*J*). Five brains shown in *A–E* (3× 101LL/263K and 2× 101LL/GSS) showing very low levels of deposition were selected for further analysis to quantify the levels of TSE infectivity and PrP^{Sc} in each tissue. Very low levels of PrP deposition were observed in brain tissue, which varied between each individual mouse brain. Deposition was mainly observed in the thalamus (*F–H*). Thalamus of an uninfected 101LL mouse is shown for background comparison (*I*). *A–E* and *J*, 4× magnification; *F–I*, 20× magnification. *A*, 101LL/263K(a); *B*, 101LL/263K(b); *C*, 101LL/263K(c); *D*, 101LL/GSS(d); *E*, 101LL/GSS(e); *F*, thalamus of 101LL/263K(a); *G*, thalamus of 101LL/263K(c); *H*, thalamus of 101LL/GSS(d); *I*, thalamus of 303-day-old uninfected 101LL mouse; and *J*, Wt/ME7 control.

TABLE 1

Tissues selected for analysis

Details of clinical disease and vacuolar pathology in the five tissues selected for analysis. All mice showed positive clinical and vacuolar signs of TSE disease and low levels of PrP deposition.

Tissue used for titration	Clinical TSE	Vacuolar pathology	PrP deposition[a]	Incubation period	
				Primary[b]	Secondary[c]
				days ± S.E.	
101LL/263K(a)	Positive	Positive	+	385	109 ± 2
101LL/263K(b)	Positive	Positive	+/−	464	129 ± 2
101LL/263K(c)	Positive	Positive	+/−	534	262 ± 4
101LL/GSS(d)	Positive	Positive	+	259	154 ± 3
101LL/GSS(e)	Positive	Positive	+/−	252	123 ± 1

[a] Scoring of PrP deposition: +++, high; ++, medium; +, low; +/−, very small grainy deposits.
[b] Incubation time of each individual mouse on primary transmission of either 263K or P102L GSS.
[c] Incubation time of 101LL mice inoculated with 1% brain homogenate from each specific 101LL/263K- or 101LL/GSS-infected tissue. Transmission of disease on subpass to 101LL mice was 100% in each case.

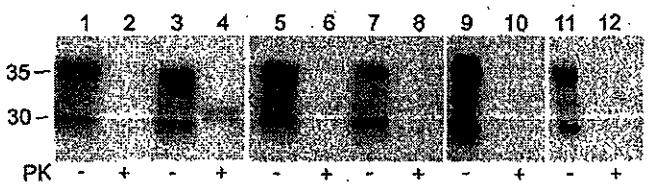

FIGURE 2. **Low or undetectable levels of PrP-res in 101LL/GSS- and 101LL/263K-infected brain.** Residual inoculum from the tissues selected for ID_{50} bioassay were analyzed by immunoblot following PK treatment to detect PrP-res. *Lanes 2, 4, 6, 8, 10,* and *12,* digested with PK at 20 μg/ml for 1 h at 37 °C; *lanes 1, 3, 5, 7, 9,* and *11,* no PK control; *lanes 1* and *2,* uninfected Wt 129/Ola mouse; *lanes 3* and *4,* 101LL/263K(a); *lanes 5* and *6,* 101LL/263K(b); *lanes 7* and *8,* 101LL/263K(c); *lanes 9* and *10,* 101LL/GSS(d); and *lanes 11* and *12,* 101LL/GSS(e). All samples were loaded at 10 mg/ml (w/v) wet weight tissue (200 μg per lane). Blots probed with mAb 8H4.

used for each series of procedures (ID_{50} determination, PK digestion, IHC, etc.). This allowed direct correlation to be made between the level of infectivity and PrP-res in each individual brain and avoided any variation that may occur between tissues, as is often observed on a primary transmission. Moreover this approach avoided the necessity of carrying out large numbers of titration experiments, which would have been both impractical and ethically unacceptable. Inocula were prepared from each individual tissue as 10% sterile saline homogenates and used to produce a series of 10-fold dilutions (10^{-2} to 10^{-9}) for inoculation. Wild-type control 129/Ola and transgenic 101LL mouse brains infected with the well characterized mouse scrapie strain ME7 (Wt/ME7 and 101LL/ME7, respectively) (18) were also assayed as controls. The seven samples were inoculated intracerebrally into groups of 129/Ola mice for Wt/ME7, and transgenic 101LL mice for all other samples. The percentage of mice that developed TSE pathology was calculated for each group in each dilution series, and the ID_{50} was determined using the Karber calculation (24). The numbers of infectious units per gram tissue (IU/g) for each individual mouse brain are shown in Table 2. Assuming a ±0.5 log error for each titer (24), all 101LL/GSS and 101LL/263K samples produced titers of infectivity ranging from ~10^7 to 10^9 IU/g. The highest titer ($10^{9.8}$) was identified in 101LL/GSS(d), however a titer of $10^{8.7}$ was also identified in 101LL/263K(a). Both of these brains showed low levels of PrP deposition by IHC, but titers were higher than that measured in control Wt/ME7 brain ($10^{8.5}$), which showed significantly more PrP deposition by IHC (Fig. 1). Titers in the other three tissues were similar ($10^{7.2}$ to $10^{7.5}$) and confirmed a high level of infectivity in the presence of extremely low or undetectable PrP deposition in the brain (Figs. 1 and 2). The results of the ID_{50} determination therefore prove the presence of high levels of infectivity in 101LL transgenic mice infected with P102L GSS or hamster 263K.

Little or No PrP-res Is Detected in Highly Infectious Tissue—IHC using anti-PrP monoclonal and polyclonal antibodies found little or no PrP deposition in brain tissue of 101LL/263K and 101LL/GSS infected mice (Fig. 1, and data not shown). However, IHC does not distinguish between different forms of PrP, therefore direct measurement of brain PrP-res levels was undertaken to determine the amount of PrP-res associated with titer of infectivity in each brain, listed in Table 1. Residual inoculum from each bioassay was mixed with detergent buffer and digested with PK (Fig. 2), and a 2-fold serial dilution from 1 mg/ml to 3.9 μg/ml (wet weight brain tissue) was analyzed by immunoblotting with mAb 8H4 (27). Recombinant PrP was loaded on each gel at 50 ng as an internal control. For the ME7-infected tissues, the limit of PrP-res detection was 15.6 μg/ml for Wt/ME7 homogenate and 31.3 μg/ml for 101LL/ME7 homogenate. Hence the same agent produced ~2-fold less PrP-