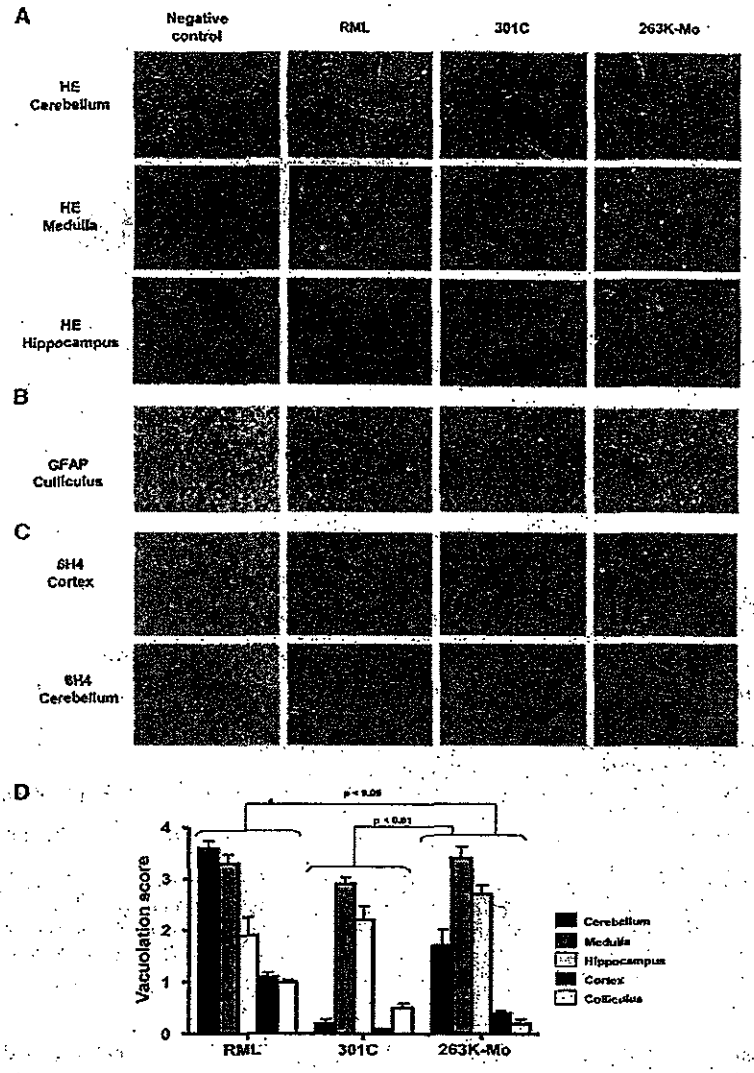

Figure 6. Histopathological Features of the Disease Induced by Inoculation of Mice with PMCA-Generated 263K-Mo PrP^{Sc}

Brains from sick mice in which disease was produced by inoculation with the newly generated 263K-Mo prions after 15 rounds of PMCA (first passage) or the known mouse strains RML and 301C were analyzed by histological studies. As a control, we used brain of a mouse inoculated with PBS and sacrificed without disease at 350 days after inoculation.

(A) Spongiform degeneration was evaluated after hematoxilin-eosin (HE) staining of three different brain areas (cerebellum, medulla, and hippocampus) and was visualized at a 40× magnification.

(B) Reactive astrogliyosis was evaluated in the inferior culliculus by staining with glial fibrillary acidic protein antibody.

(C) PrP accumulation in these animals was evaluated in the occipital cortex and cerebellum by staining of the tissue with the 6H4 antibody.

(D) The vacuolation profile in each brain area was estimated with a semiquantitative scale, as described in the Experimental Procedures. The brain areas used were the following: occipital cortex, cerebellum (mostly white matter), medulla (spinal 5 nucleus, interpolar part), inferior colliculum, and hippocampus (CA1 and CA2 regions). We also included in the analysis brain sections from animals inoculated with RML and 301C. The values represent the average ± standard error of the extent of vacuolation from the five animals analyzed in each set. Statistical analysis by two-way ANOVA with brain regions and prion origin as the variables indicated that differences were highly significant ($p < 0.001$). To assess the significance of the differences between each known prion strain and 263K-Mo, we used the Dunnett multiple comparison post-test, and the p values for each combination are shown.

origin of CWD and its potential to transmit to humans are currently unknown. This is worrisome, considering that CWD has became endemic in some parts of the USA and that the number of cases continues to increase (Williams, 2005). CWD transmissibility studies have been performed in many species in order to predict how this disease could be spread by the consumption of CWD meat (Sigurdson and Aguzzi, 2006). Transmission of CWD to humans cannot be ruled out at present, and a similar infective episode to BSE involving CWD could result in catastrophic consequences.

The exciting scientific problem coupled with the relevant public-health issue prompted us to develop strategies to reproduce the species-barrier phenomenon in the test tube. We reported previously the generation of infectious prions in vitro by cyclic replication of the protein misfolding process featuring the pathogenesis of prion diseases (Castilla et al., 2005). These results were reproduced and extended by other groups to better dissect the elements required for prion replication (Deleault et al., 2007; Weber et al., 2007). The PMCA technology has been adapted to replicate prions from various species (Deleault et al., 2005; Jones et al., 2007; Kurt et al., 2007; Murayama et al., 2007; Safaroff et al., 2005; Soto et al., 2005) and even to use bacterially produced recombinant PrP as substrate (Atarashi et al., 2007). The conclusion drawn from these studies together with the findings reported in this manuscript is that propagation of the PrP^{Sc} misfolding results in formation of infectious material, which maintains the strains and species-barrier properties of the original prions. Qualitatively similar conclusions have been obtained for yeast prions, which are a group of "infectious proteins" that behave as a non-Mendelian genetic element and transmit biological information in the absence of nucleic acid (Wickner et al., 1995). Recent studies showed that bacterially produced N-terminal fragments of the yeast prions Sup35p and Ure2p when transformed into amyloid fibrils were able to propagate the prion phenotype to yeast cells (Brachmann et al., 2005; King and Diaz-Avalos, 2004; Tanaka et al., 2004). Infection of yeast with different conformers led to generation of distinct prion strains in vivo (Brachmann et al., 2005; Tanaka et al., 2004). Remarkably, yeast prions also show the species-barrier phenomenon, and recent data indicate that strain conformation is the critical determinant of cross-species prion transmission (Tanaka et al., 2005).

In the current study, we demonstrate the generation of new infectious prions across the species barrier. For this purpose, we

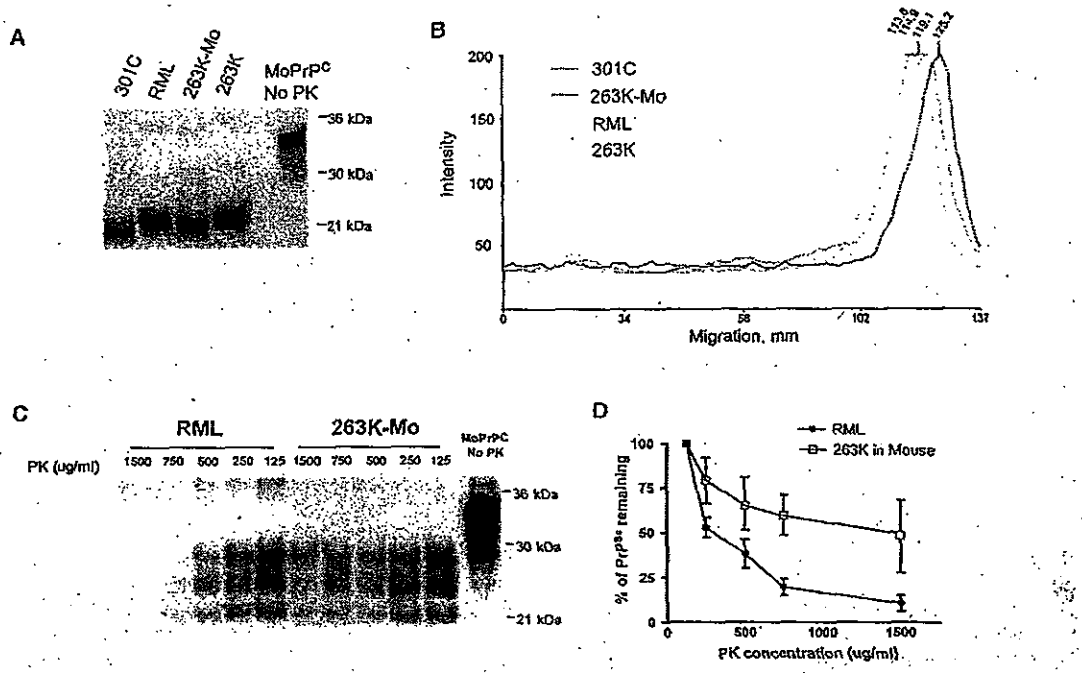

Figure 7. Biochemical Characteristics of 263K-Mo PrP^{Sc}

(A) Samples from brains of mice inoculated with 263K-Mo, RML, or 301C were used to study the electrophoretical migration after deglycosylation and PK treatment.

(B) For assessment of the electrophoretical differences among distinct strains, the blot in (A) was scanned and analyzed by software included in the UVP image analysis system to locate the exact position of the bands.

(C) The PK resistance profile of 263K-Mo PrP^{Sc} was studied and compared with RML.

(D) The results of the experiment shown in (B) were quantitated by densitometric analysis. The data in the figure represent the average ± standard error from three independent animals. The differences were statistically significant as evaluated by one-way ANOVA ($p < 0.01$).

mixed PrP^{Sc} from one species with PrP^{C} from a different animal species and subjected the mixture to serial rounds of PMCA to generate, propagate, and stabilize new prion strains. Hamster PrP^{Sc} generated from mouse RML prions was infectious to wild-type hamsters. Detailed analysis of the disease characteristics and comparison with the illness produced by several known hamster prion strains indicate that the in vitro-generated infectious material across the species barrier corresponds to a new prion strain in hamsters (termed RML-Ha). The main differences of the RML-Ha were on the incubation times after i.p. inoculation, the extremely high resistance to PK degradation, and the pattern of brain damage (Table S1). Similarly, PrP^{Sc} generated by conversion of mouse PrP^{C} with hamster PrP^{Sc} from the 263K strain was shown to be infectious to wild-type mice, with an incubation period comparable to that obtained after inoculation with some of the mouse-adapted scrapie strains, such as RML. Again, the disease produced by the new prions (termed 263K-Mo) was clearly distinguishable from the one produced by some of the currently known mouse prion strains. The major differences were seen in the electrophoretical migration, extremely high resistance to proteolytic degradation, and pattern of brain spongiform degeneration (Table S2). To rule out that newly generated PrP^{Sc} in these experiments was coming from "de novo" spontaneous conversion of PrP^{C} into PrP^{Sc} during PMCA, we used samples of healthy brain homogenate from ten different mice and hamsters that were subjected to serial rounds of PMCA amplification in the absence of PrP^{Sc} seed. After up to 20 serial rounds of PMCA, we did not observe de novo formation of PrP^{Sc} in any of the samples. This material was inoculated into wild-type animals, and no disease was observed more than 400 days after inoculation. These results strongly indicate that the generation of PrP^{Sc} reported in the present study was due to interspecies prion conversion. Nevertheless, we would like to highlight that recently we have been able to generate in vitro PrP^{Sc} de novo without addition of PrP^{Sc} seed (data not shown). However, to reach this aim, the PMCA conditions need to be modified. The modifications include changes on the PMCA parameters (length of incubation and potency of sonication), preincubation, or pretreatment of the normal brain homogenate to induce/stabilize PrP misfolding prior to PMCA. These findings suggest that de novo formation of PrP^{Sc} can be experimentally distinguished from replication of preformed PrP^{Sc}, indicating that the biochemical, conformational, or stability properties of the PrP structures involved in both processes are probably different. Standard PMCA conditions, as those used in the current study, do not result in spontaneous PrP^{Sc} formation.

Interestingly, in our serial PMCA amplifications of RML PrP^{Sc} into hamster PrP^{C}, we observed a progressive change on the western blot profile of the newly generated RML-Ha PrP^{Sc}. Indeed, in the first round of PMCA, the glycoform distribution

pattern was reminiscent of RML and later switched to a profile typical of the hamster strains, characterized by the predomination of the diglycosylated form (Figure 1D). Our interpretation of this result was that consecutive rounds of PMCA may enable the new prion strain to adapt and stabilize. To further study this possibility in our experiments in which mouse prions were generated from 263K hamster prions, we inoculated the material generated after various rounds of PMCA. Strikingly, similar amounts of PrP^{Sc} generated after one and three rounds of PMCA produced disease with incomplete attack rates and/or very long incubation periods (Figures 5B and 5C). Incubation time stabilized after six rounds of serial PMCA, suggesting that at this point the new strain is fully adapted. These findings suggest that PMCA is not only able to reproduce the interspecies transmission of prions but is also able to mimic the strain adaptation process observed in vivo. In vivo adaptation and stabilization of prions generated after crossing the species barrier takes at least four consecutive passages, which requires several years of work (Race et al., 2001, 2002). Conversely, strain adaptation by PMCA takes only 2 or 3 weeks. Importantly, the kinetics of adaptation in vitro and in vivo, as well as the characteristics of the stabilized material, are very similar. Indeed, it has been reported that three serial passages of 263K in mice produce disease in all animals, with an incubation time of around 300 days (Race et al., 2002). This result is very similar to the data obtained with the material generated in vitro after three successive rounds on PMCA replication (Figures 5B and 5C). Moreover, less than three in vivo passages produced an incomplete attack rate, and more than three passages are needed to obtain a stable and low incubation period (Race et al., 2002), which is in the same range of our 263K-Mo infectious material. Finally, similar to our in vitro data, the in vivo cross-species transmission between hamsters and mice also led to the generation of unique prion strains (Race et al., 2001, 2002). Although we are tempted to speculate that each PMCA round has the same effect on strain adaptation as did each in vivo passage, more experiments with other species combinations are needed to reach this conclusion.

In summary, our results show that all elements controlling interspecies transmission of prions are contained in a cell-free system and that new prion strains can be generated, adapted, and stabilized upon crossing the species barrier in vitro by PMCA. These findings provide additional support for the prion hypothesis, suggesting that species-barrier transmission and strain generation are determined by the propagation of PrP misfolding. Furthermore, the data demonstrate that PMCA is a valuable tool for the investigation of the strength of the barrier between diverse species, its molecular determinants, and the expected features of the new infectious material produced. Finally, our findings suggest that the universe of possible prions is not restricted to those currently known but that likely many unique infectious foldings of the prion protein may be produced and that one of the sources for this is cross-species transmission.

EXPERIMENTAL PROCEDURES

Preparation of Tissue Homogenates

Healthy and sick animals were perfused with phosphate-buffered saline (PBS) plus 5 mM ethylenediaminetetraacetic acid (EDTA) before the tissue was harvested. Ten percent brain homogenates (w/v) were prepared in conversion buffer (PBS containing 150 mM NaCl, 1.0% Triton X-100, and the complete cocktail of protease inhibitors from Boehringer Mannheim, Mannheim, Germany). The samples were clarified by a brief, low-speed centrifugation (1500 rpm for 30 s) with an Eppendorf centrifuge (Hamburg, Germany), model 5414.

Serial Replication of Prions In Vitro by PMCA

Aliquots of 10% brain homogenate from clinically sick mice infected with RML or 301C and hamsters infected with 263K, HY, or DY prions were diluted into 10% hamster or mouse healthy brain homogenate. Samples were loaded onto 0.2 ml PCR tubes and positioned on an adaptor placed on the plate holder of a microsonicator (Misonix Model 3000, Farmingdale, NY). Each PMCA cycle consisted of 30 min incubation at 37°C followed by a 20 s pulse of sonication set at potency of 7. Samples were incubated without being shaken immersed in the water of the sonicator bath. After a round of PMCA cycles, a 10 µl aliquot of the amplified material was diluted into 90 µl of more normal brain homogenate, and a new round of PMCA cycles was performed. This procedure was repeated several times to reach the final dilutions indicated in the text. The detailed protocol for PMCA, including reagents, solutions, and troubleshooting, has been published elsewhere (Castilla et al., 2006; Saa et al., 2005).

Proteinase K Degradation Assay

The standard procedure for digestion of PrP^{Sc} consists of subjecting the samples to incubation in the presence of PK (50 µg/ml) for 60 min at 37°C. The digestion was stopped by addition of electrophoresis sample buffer, and the protease-resistant PrP was revealed by western blotting. So that the profile of PK sensitivity for in vitro- and in vivo-generated PrP^{Sc} could be studied, the samples were incubated for 60 min at 37°C with different concentrations of PK ranging from 0 to 2500 µg/ml. The PK_{50} values represent the concentration of PK needed to digest half of the protein, and these values are estimated on the basis of the densitometric analysis of three replicated western blots.

Guanidine Denaturation Assay

Samples were incubated with different concentrations of guanidine hydrochloride for 2 hr at room temperature with shaking. Thereafter, samples were incubated in the presence of 10% sarkosyl for 30 min at 4°C and centrifuged at 100,000 × g for 1 hr in a Biosafe Optima MAX ultracentrifuge (Beckman Coulter, Fullerton, CA). The pellet of the centrifugation was resuspended in conversion buffer and treated with PK as described above. Equivalent aliquots of pellet were analyzed by western blot. The Gdn_{50} value corresponds to the concentration of guanidine hydrochloride required to denaturate 50% of the protein, and these values were estimated on the basis of the densitometric analysis of three replicated western blots.

Protein Deglycosylation Assay

PrP^{Sc} samples were first digested with PK as describe above. After addition of 10% sarkosyl, samples were centrifuged at 100,000 × g for 1 hr at 4°C, supernatant was discarded, and the pellet resuspended in 100 µl of glycoprotein denaturing buffer (New England Biolabs, Beverly, MA) and incubated for 10 min at 100°C. Thereafter, 26 µl of 50 mM sodium phosphate (pH 7.5) containing 1% nonidet P-40 and 3 µl of peptide N-glycosidase F (New England Biolabs, Beverly, MA) were added. Samples were incubated for 2 hr at 37°C, and the reaction was stopped by the addition of electrophoresis buffer and samples were analyzed by western blot.

Western Blot

Proteins were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, electroblotted into nitrocellulose membrane, and probed with 6H4 (for mouse samples) and 3F4 (for hamster samples) antibodies at a 1:5000 dilution. The immunoreactive bands were visualized by enhanced chemoluminesence assay (Amersham, Piscataway, NJ) with an UVp image analysis system. So that the quantity of PrP^{Sc} in the western blot would be assessed, densitometric analyses were done by triplicate.

PrP^{Sc} Quantification

To inject the same quantity of PrP^{Sc} from each preparation, we compared the samples by western blotting after PK digestion. To obtain a reliable and robust quantification, we ran several different dilutions of the sample in the same gel, to avoid artifacts due to saturation of the signal or to too weak of a signal.

Infectivity Studies

In vivo infectivity studies were done in C57Bl6 female mice or Golden Syrian female hamsters, purchased from Charles river. Animals were 4 to 6 weeks old at the time of inoculation. Anesthesized animals were injected stereotaxically into the right hippocampus with 2 or 4 µl of the mouse or hamster infectious material, respectively. For the i.p. infectivity studies, 100 µl of the sample were injected into the peritoneal cavity. The quantity of infectious material injected corresponds to the plateau portion of the incubation period; therefore, small differences in the amount of infectivity should not change incubation period unless there are strain differences. The onset of clinical disease was measured by scoring of the animals twice a week. For mice, the following scale was used: 1, normal animal; 2, roughcoat on limbs; 3, extensive roughcoat, hunckback, and visible motor abnormalities; 4, urogenital lesions; and 5, terminal stage of the disease in which the animal presented with cachexia and lies in the cage with little movement. For hamsters, the following scoring scale was used: 1, normal animal; 2, mild behavioral abnormalities including hyperactivity and hypersensitivity to noise; 3, moderate behavioral problems including tremor of the head, ataxia, wobbling gait, head bobbing, irritability, and aggressiveness (or lethargy in case of the DY strain); 4, severe behavioral abnormalities including all of the above plus jerks of the head and body and spontaneous backrolls; and 5, terminal stage of the disease in which the animal lies in the cage and is no longer able to stand up. Animals scoring level 4 during two consecutive weeks were considered sick and were sacrificed to avoid excessive pain via exposition to carbonic dioxide. Brains were extracted, the right cerebral hemisphere was frozen and stored at −70°C for biochemical examination of PrP^{Sc} with western blots, and the left hemisphere was used for histology analysis.

Histopathological Studies

Brain tissue was fixed in 10% formaldehyde solution, cut in sections, and embedded in paraffin. Serial sections (6 µm thick) from each block were stained with hematoxylin-eosin, or incubated with monoclonal antibodies recognizing PrP or the glial fibrillary acidic protein, via our previously described protocols (Castilla et al., 2005). Samples were visualized with a Zeiss microscope. The vacuolation profile was estimated by consideration of both number and size of spongiform degeneration in five different brain areas: occipital cortex, cerebellum (mostly white matter), medulla (spinal 5 nucleus, interpolar part), inferior colliculum, and hippocampus (CA1 and CA2 regions). Each analyzed brain area was scored from 0 to 4 according to the extent of vacuolation in slides stained with hematoxilin-eosin and visualized at a 40× magnification. Samples were analyzed blindly by two different persons, and the scores represent the average of the two determinations.

Statistical Analysis

The differences in incubation periods, histopathological profile of brain damage, and biochemical characteristics of PrP^{Sc} were analyzed by ANOVA, followed by the Dunnett Multiple Comparison post-test to estimate the significance of the differences between the newly generated strains and each of the other hamster and mouse prion strains studied. For these studies, the data were analyzed with the GraphPad Instat, version 3.05 software.

SUPPLEMENTAL DATA

Supplemental Data include one figure and two tables and can be found with this article online at http://www.cell.com/cgi/content/full/134/5/757/DC1/.

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研究報告の概要

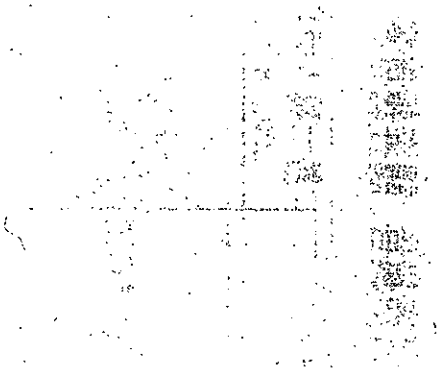
○伝達性海綿状脳症感染性の尿への排出
伝達性海綿状脳症の自然感染経路は未だに仮説段階である。TSE暴露の潜在的要因としての尿を調査するため、高感度検出法とTSE感染性の定量を行った。263Kスクレイピーの臨床症状を呈しているハムスター22匹からプールした尿は、3.8 ± 0.9 感染量/mLの感染性を含んでいた。同じ動物由来の腎臓と膀胱のホモジネートの滴定は2万倍の濃度を示した。これら同じ組織の組織学的、免疫組織化学的分析では、腎臓における疾患関連プリオンタンパク質の散発的な沈着を除いて、炎症あるいは他の病変は見られなかった。尿におけるTSE感染源は未だに解明されていないが、これらの結果は、TSE感染性が尿中に排出されており、その結果自然のTSEの水平感染において何らかの役割を果たしていることを立証している。また、ヒトの尿由来ホルモンや他の医薬品からのTSE伝播の潜在的リスクを示している。

報告企業の意見	今後の対応
263Kスクレイピーの臨床症状を呈しており、腎臓・膀胱に炎症のないハムスター22匹からプールした尿にTSEの感染性があることが示されたとの報告である。	今後も引き続き、プリオン病に関する新たな知見及び情報の収集に努める。

使用上の注意記載状況・その他参考事項等

新鮮凍結血漿「日赤」
新鮮凍結血漿-LR「日赤」

血液を介するウイルス、
細菌、原虫等の感染
vCJD等の伝播のリスク



RESEARCH

Excretion of Transmissible Spongiform Encephalopathy Infectivity in Urine

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The route of transmission of most naturally acquired transmissible spongiform encephalopathy (TSE) infections remains speculative. To investigate urine as a potential source of TSE exposure, we used a sensitive method for detection and quantitation of TSE infectivity. Pooled urine collected from 22 hamsters showing clinical signs of 263K scrapie contained 3.8 ± 0.9 infectious doses/mL of infectivity. Titration of homogenates of kidneys and urinary bladders from the same animals gave concentrations 20,000-fold greater. Histologic and immunohistochemical examination of these same tissues showed no indications of inflammatory or other pathologic changes except for occasional deposits of disease-associated prion protein in kidneys. Although the source of TSE infectivity in urine remains unresolved, these results establish that TSE infectivity is excreted in urine and may thereby play a role in the horizontal transmission of natural TSEs. The results also indicate potential risk for TSE transmission from human urine–derived hormones and other medicines.

Transmissible spongiform encephalopathies (TSEs) are fatal neurologic diseases. In humans, a long asymptomatic incubation period is followed by a progressive clinical course that typically lasts a few months to a year. TSE infectivity and pathologic changes are concentrated in the nervous system; however, much of the transmission risk results from parenteral exposure to the much lower concentrations of infectivity found in tissues outside the nervous system. Thus, despite the very low concentration of TSE infectivity in blood (*1,2*), 4 human cases of transmission of variant Creutzfeldt-Jakob disease through blood transfusions have been documented (*3,4*). If TSE infectivity were excreted, human urine, which is a source of injectible fertility hormones and other drugs (*5,6*), could also pose a risk for transmission. Infected urine might also account for the horizontal transmission of sheep scrapie and might contribute to the natural spread of chronic wasting disease in deer and elk.

Early attempts to transmit Creutzfeldt-Jakob disease by cross-species inoculation of rodents and primates with urine from diseased patients failed (*7,8*). More recent attempts in which urine from infected hamsters was injected back into hamsters have produced variable results (*9,10*). Two other studies have reported infectivity in urine (*11*) and infectivity with disease-specific prion protein (PrP^d) in kidneys of mice with simultaneous scrapie and nephritis but not in those with scrapie alone (*12*). To resolve these discrepancies, we used a highly sensitive and precise method of measuring low concentrations of TSE infectivity, which we have successfully used for quantitation of TSE infectivity in blood (*1,2*), to measure the concentration of TSE infectivity in urine of scrapie-infected hamsters.

Materials and Methods

Urine Collection and Processing

Urine was collected from a cohort of 22 Syrian hamsters (Harlan Sprague-Dawley, Haslet, MI, USA) that had been infected by intracranial injection with 10% (wt/vol) scrapie brain homogenate (263K strain) and from a cohort of 8 age-matched, noninoculated control animals. At the time of urine collection, the scrapie-infected hamsters showed clear clinical evidence of disease but were still able to drink and eat (67–74 days postinoculation). Hamsters were placed 2 at a time for 24 hours in metabolism cages in which they had access to water but not food. Food was

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