

Rabbits are not resistant to prion infection

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The ability of prions to infect some species and not others is determined by the transmission barrier. This unexplained phenomenon has led to the belief that certain species were not susceptible to transmissible spongiform encephalopathies (TSEs) and therefore represented negligible risk to human health if consumed. Using the protein misfolding cyclic amplification (PMCA) technique, we were able to overcome the species barrier in rabbits, which have been classified as TSE resistant for four decades. Rabbit brain homogenate, either unseeded or seeded in vitro with disease-related prions obtained from different species, was subjected to serial rounds of PMCA. De novo rabbit prions produced in vitro from unseeded material were tested for infectivity in rabbits, with one of three intracerebrally challenged animals succumbing to disease at 766 d and displaying all of the characteristics of a TSE, thereby demonstrating that leporids are not resistant to prion infection. Material from the brain of the clinically affected rabbit containing abnormal prion protein resulted in a 100% attack rate after its inoculation in transgenic mice overexpressing rabbit PrP. Transmissibility to rabbits (>470 d) has been confirmed in 2 of 10 rabbits after intracerebral challenge. Despite rabbits no longer being able to be classified as resistant to TSEs, an outbreak of “mad rabbit disease” is unlikely.

in vitro replication | scrapie | transmissible spongiform encephalopathy

Transmissible spongiform encephalopathies (TSEs), or prion diseases, are infectious neurodegenerative disorders affecting humans and animals that are invariably fatal and with no currently available treatments for prevention or cure. The misfolded version (PrP^{Sc}) of the host cellular prion protein (PrP^C) is the infectious agent (1), but additional fundamental aspects of TSEs, such as what dictates the generation of strains and the transmission barrier, remain elusive. The species barrier determines the ability of certain prion strains to infect some species but not others. Unsuccessful experimental challenges to overcome this species barrier and lack of documented natural TSE cases in certain families, including leporids, equids, and canids, have led to the belief that they are resistant to TSEs. Several experimental approaches have been used in an attempt to overcome the species barrier and to reduce incubation times. These include the use of transgenic mice, which overexpress PrP^C, making them more susceptible to certain prion infections when compared with the original species, and intracerebral instead of oral inoculation, with the former being invariably more efficient (2). Such methodologies were the only way to investigate the species barrier even though they differ from natural transmission.

The protein misfolding cyclic amplification (PMCA) technique (3) is a powerful in vitro tool that mimics the in vivo conversion process of PrP^C to PrP^{Sc} but with accelerated kinetics, amplifying minute quantities of any PrP^{Sc} present in a sample in an exponential fashion (4–7). This technique, which considerably reduces the protracted incubation periods, is potentially very important in the prion field in general (8–10), and more specifically, for the study of the transmission barrier (5, 11). Using our experience in replicating prions in vitro that can subsequently be used to infect susceptible hosts (9, 11, 12–16), we wanted to determine whether

the lack of any documented natural cases or successful in vivo challenges was sufficient to justify rabbits being considered resistant to prion disease. We chose the rabbit as a TSE-resistant model because it is a laboratory animal, is commonly eaten by humans, is commercially reared, and is abundant in wild populations occurring worldwide and cohabiting with potential TSEs carriers, such as sheep and cattle in Europe, and deer in the United States and Canada.

With PMCA, we have shown, in rabbits, that a species previously classified as resistant to prion disease is both susceptible to and capable of developing clinically transmissible prion disease.

Results

In Vitro Generation of Rabbit PrP^{res}. Unseeded normal rabbit brain homogenate as well as normal rabbit brain homogenates seeded in vitro with different scrapie mouse adapted prion strains (ME7, RML, 22F, 79A, 139A, and 22L), sheep scrapie (SSBP/1 and CH1641), bovine spongiform encephalopathy (BSE), and chronic wasting disease (CWD) were subjected to serial automated PMCA (saPMCA) in an attempt to compare the ability of generating rabbit PrP^{res} (RaPrP^{res}). Bearing in mind that saPMCA is not a quantitative technique, rabbit PrP appeared to be quite susceptible to protein misfolding, as all seeds were able to generate proteinase-K (PK) resistant RaPrP^{res} by round 7 and the unseeded homogenate produced RaPrP^{res} by round 13, suggesting that it may be transmissible in vivo (Table 1 and Fig. 1).

On this basis, we selected saPMCA product from the unseeded normal rabbit brain homogenate, which we refer to as de novo, and the product from the homogenate seeded with the well-characterized ME7 scrapie strain, a mouse adapted prion strain that has never been transmitted successfully in previous challenges to rabbits (17), as challenge inocula in vivo. Both in vitro-derived RaPrP^{res} products were easily amplified further in vitro (Fig. S1).

Infectivity Studies. Ten 3-mo-old male New Zealand White rabbits (HsdJf: NZW, Harlan, The Netherlands), of identical PrP genotype, were randomly divided into three groups and challenged intracerebrally with either de novo RaPrP^{res} ($n = 3$), ME7 RaPrP^{res} ($n = 3$) or original (murine) ME7 mouse brain homogenate ($n = 4$). Negative control material was obtained from three additional unchallenged New Zealand White rabbits. After 766 dpi, one animal from the group challenged with de novo RaPrP^{res} developed clinical neurological signs, principally hind limb ataxia and dullness (Movies S1 and S2) and died 4 d later.

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Table 1. Rounds (R1-R14) of serial automated PMCA using rabbit brain homogenate as substrate

Seed	Species	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11	R12	R13	R14
RML	Mouse														
ME7	Mouse														
22L	Mouse														
139A	Mouse														
79A	Mouse														
22F	Mouse														
SSBP/1	Sheep														
CH1641	Sheep														
CWD	Deer														
BSE	Cow														
Unseeded	Mouse														

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

Not determined 0% 25% 50% 75% 100%

Biochemical analysis of the brain by Western blotting (WB) confirmed the presence of PrP^{Sc} (Fig. 2) showing characteristic bands with different glycosylation and electrophoretic migration patterns compared with other known PrP^{Sc} profiles (Fig. 2A). This *in vivo* pattern was very similar to that previously observed in the material generated *in vitro* (Fig. 2B). To characterize the strain generated *in vivo*, we performed comparative tests with two other prion strains: RML and ME7. The *de novo* RaPrP^{Sc} showed a greater resistance to PK treatment and guanidine denaturation (Fig. 3). Histological examination of the brain showed vacuolation of the neuropil and the cytoplasm of neurons. Immunohistochemistry (IHC) showed astrocytosis and the presence of PrP^{Sc}, and PET-blot analysis also revealed PrP^{res} (PK resistant PrP). These findings were strikingly similar to those observed in classical cases of TSEs (Fig. 4).

The ability of RaPrP^{Sc} to infect cells *in vitro* was tested using a mouse PrP knockout cell line (CF10) (18) expressing rabbit PrP^C. The cells were exposed to brain homogenate derived from the clinically positive rabbit inoculated with *de novo* RaPrP^{Sc}, passaged five times, and analyzed for PrP^{Sc}. PrP^{Sc}, probably representing *de novo* RaPrP^{Sc} from the initial inoculum, was detected at passages 0 and 1 but not at passages 3, 5 (Fig. S24), and 12. The general susceptibility of the cells to prion infection was confirmed by the ability of CF10 cells to produce PrP^{Sc} over multiple passages following exposure to 22L mouse scrapie infectivity (Fig. S2B). Thus, the inability of *de novo* RaPrP^{Sc} to trigger PrP^{Sc} formation suggested that it was unable to persistently infect the cells.

After 818 d post inoculation (dpi), one animal challenged with ME7 RaPrP^{res} died without clinical signs 1 day after being moved to a new cage. Histological and biochemical analysis of the brain from this animal failed to show any lesions typical of TSE or PrP^{Sc}. However, PMCA amplification of this sample using normal rabbit brain homogenate as a substrate revealed glycosylation bands and electrophoretic migration patterns characteristic of a TSE (Fig. S3). PMCA data generated using serially diluted *de novo* RaPrP^{Sc} as a control showed the levels from the ME7 RaPrP^{Sc} challenged rabbit to be 10³ to 10⁴ times less than the *de novo* RaPrP^{Sc} case (Fig. S3). This low quantity could explain the failure of IHC, WB or Pet-Blot to detect PrP^{Sc}. Two additional rabbits had to be culled because of intercurrent diseases, one rabbit from the ME7 group and one from the ME7 RaPrP^{Sc} group, at 1,078 and 1,085 dpi, respectively; both were negative for PrP^{Sc} expression by WB and IHC.

At the time of writing (~1,380 dpi), all of the remaining rabbits were clinically healthy and devoid of any signs of TSE infection.

Transmission Studies. Three 8-wk-old male transgenic mice over-expressing rabbit PrP (raPrPTg) were challenged intracerebrally with a 1% (wt/vol) brain homogenate prepared from the clinically affected rabbit containing abnormal prion protein. Five raPrPTg mice were inoculated with 1% (wt/vol) healthy brain

homogenate as negative controls. The three prion-inoculated raPrPTg mice started showing clinical prion disease at a mean (\pm SEM) of 266 \pm 7 dpi and were killed at 253, 262, and 283 dpi. Biochemical analysis of their brains by WB confirmed the presence of PrP^{Sc} with similar characteristic bands seen in the *de novo* RaPrP^{Sc} sample (Fig. 5A). The raPrPTg mice used as negative controls remain healthy after 350 dpi, with the exception of one animal dying at 281 dpi, but showing no PrP^{Sc} by WB.

Transmissibility in rabbit has been studied through a second passage experiment performed in New Zealand White rabbits (HsdIif: NZW, Harlan, The Netherlands). Ten 3-mo-old male

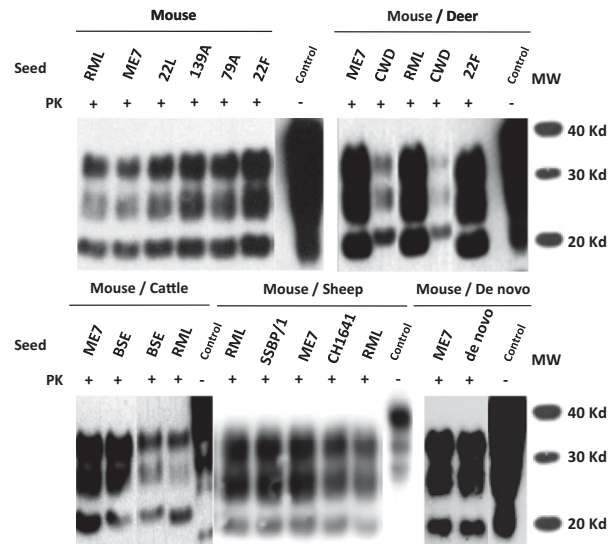


Fig. 1. Biochemical analysis of different rabbit PrP^{res} (RaPrP^{res}) generated *in vitro* by serial automated PMCA (saPMCA) using a New Zealand White rabbit brain homogenate as substrate. Rabbit brain homogenates seeded with different prion strains (mouse: RML, ME7, 22L, 139A, 79A and 22F, mule deer: CWD, cattle: BSE, sheep: SSBP/1 and CH1641) or unseeded (*de novo*) were subjected to saPMCA. Seeded samples from round 10 and the unseeded sample from round 20 were digested with 100 μ g/mL of proteinase K (PK) and analyzed by Western blot using monoclonal antibody D18. Three differential electrophoretic migration patterns are shown, depending on the seed that was used: (i) the pattern for the unglycosylated band that migrated the farthest (18–19 K_d, sheep and mouse adapted scrapie strains); (ii) the intermediate migration pattern for the same band (19–20 K_d, BSE); and (iii) the migration pattern for the unglycosylated band that migrated the least (20–21 K_d, CWD). RML and ME7 RaPrP^{res} samples are used in all blots as reference for determining the different electrophoretic migration patterns. Control, normal rabbit brain homogenate.

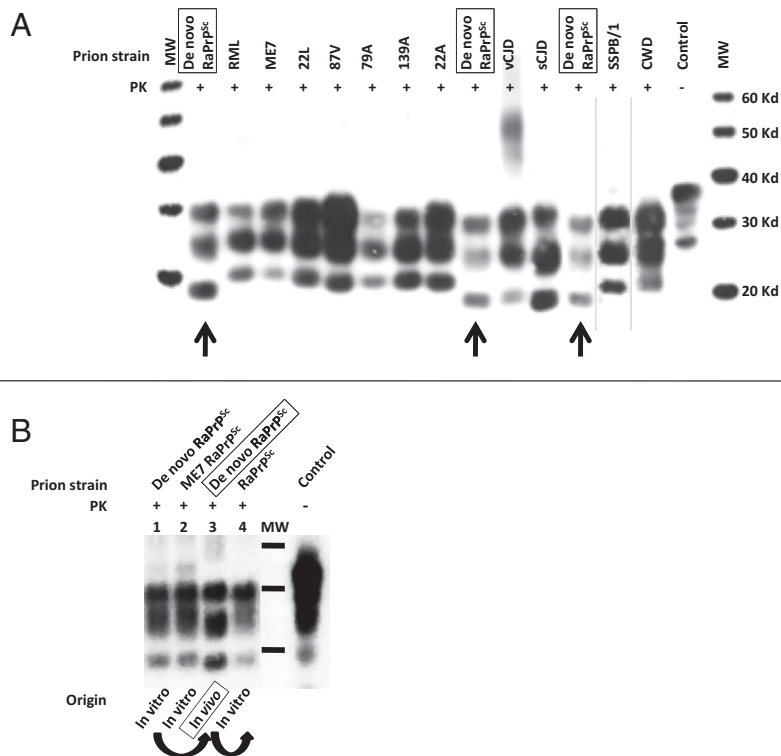


Fig. 2. Comparative electrophoretic patterns from different prion strains. (A) Brain homogenate from de novo RaPrP^{Sc} inoculated rabbit was compared biochemically with the most typical prion strains from different species. To have a clear reference and accurate comparison of the different electrophoretic migration patterns, de novo RaPrP^{Sc} was run three times (arrows). De novo RaPrP^{Sc} shows unique biochemical characteristics determined by electrophoretic migration pattern and glycosylation profile in that the unglycosylated band migrates further compared with all other strains with the exception of sporadic Creutzfeldt-Jakob disease (sCJD). However, sCJD can be easily distinguished from the RaPrP^{Sc} strain because of its glycosylation profile and the specific migration of the diglycosylation band, which does not migrate as far. (B) In vitro generated de novo and ME7 RaPrP^{Sc} (lanes 1 and 2, respectively) are biochemically compared with de novo RaPrP^{Sc} generated in vivo (lane 3). Lane 4 shows the biochemical pattern of RaPrP^{Sc} generated in vitro by PMCA, using in vivo de novo RaPrP^{Sc} as seed. Although not identical, the in vivo pattern (lane 3) was very similar to that previously observed in the material generated in vitro (lanes 1, 2, and 4). All samples were digested with 100 μ g/mL of proteinase K and were analyzed by Western blot using monoclonal antibody D18. Curved arrows indicate in vitro passage by PMCA. Control, normal rabbit brain homogenate.

NZW rabbits were randomly divided into two groups and challenged intracerebrally with the 10^{-1} (wt/vol; $n = 5$) or 10^{-3} (wt/vol; $n = 5$) brain homogenate prepared from the clinically

affected rabbit containing abnormal prion protein. After 477 dpi, one animal belonging to the 10^{-3} group died without apparent clinical signs. Biochemical analysis of the brain by WB confirmed

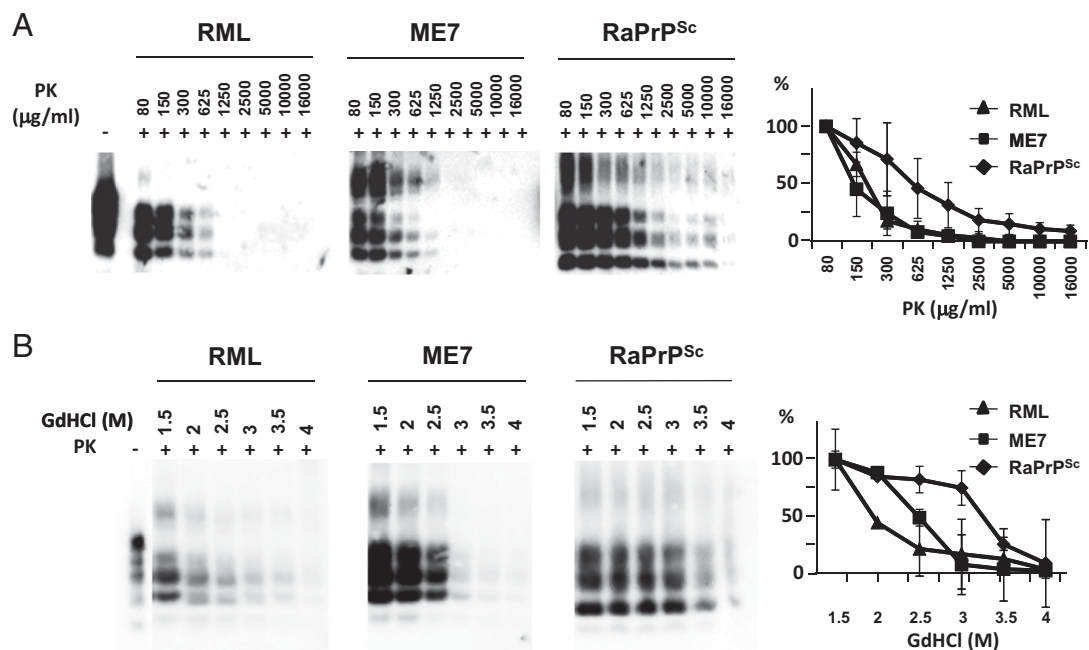


Fig. 3. Proteinase K and guanidine denaturation studies using an in vivo rabbit prion strain. (A) De novo RaPrP^{Sc} inoculated rabbit brain homogenate was subjected to several different PK concentrations (80–16,000 μ g/mL) at 42 $^{\circ}$ C for 1 h. ME7 and RML, two well-characterized prion strains, were used as reference. Although the mouse adapted scrapie strains showed a similar PK resistance, the rabbit prion strain showed a much higher resistance to PK treatment. (B) De novo RaPrP^{Sc} inoculated rabbit brain homogenate was denatured with different guanidine (GdHCl) concentrations (1.5–4 mol/L) and then subjected to standard Proteinase K (PK) digestion. ME7 and RML prion strains were used as reference. The rabbit prion showed a greater resistance to guanidine denaturation compared with RML and ME7, which had a low or intermediate resistance respectively. Graphs represent an evaluation of Western blots by densitometric analysis from three independent experiments (mean \pm SE). All samples were analyzed by Western blot using monoclonal antibody D18.

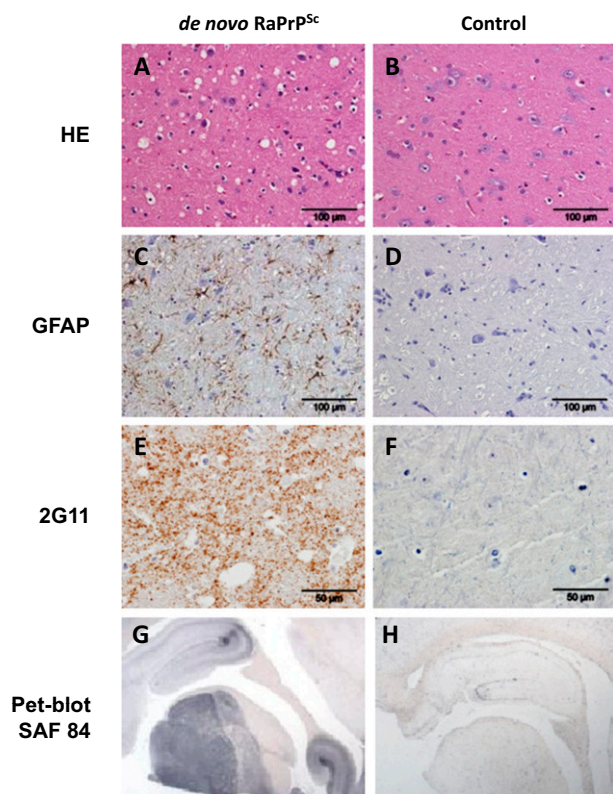


Fig. 4. Histological and immunohistochemical studies. Sections of midbrain from a rabbit challenged with de novo RaPrP^{Sc} (A, C, E, and G) and a negative control unchallenged rabbit (B, D, F, and H). Hematoxylin and eosin-stained sections showed vacuolation, predominantly in the neuropil of the brain of the challenged rabbit (A) but none in the negative control animal (B). Immunohistochemistry using antibodies to GFAP (glial fibrillary acidic protein) and abnormal prion protein (2G11) showed respectively astrocytosis (C, brown pigment) and positive labeling of PrP^{Sc} (E, brown pigment) in the brain of the challenged rabbit. Neither astrocytosis (D) nor PrP^{Sc} (F) was found in the brain of the negative control rabbit. PET-Blot analysis using monoclonal antibody SAF84 for PrP^{Sc} was positive in the challenged rabbit (G, black pigment) but was absent in the negative control (H).

the presence of PrP^{Sc} (Fig. 5B), which was indistinguishable from that previously observed in the material generated in the first passage (Fig. 2B). A second rabbit, this time from the 10⁻¹ group, showed pododermatitis and loss of weight and had to be

culled at 540 dpi for ethical reasons. Similarly, WB confirmed the presence of PrP^{Sc} in all of the brain areas examined (Fig. 5B). The rest of the rabbits remained clinically normal at ~560 dpi.

Discussion

Slightly more than 25 y ago, cattle were considered free of prion diseases. No one would have predicted the BSE epidemic with the considerable human and animal health repercussions, and the political and economic impacts that it had in Europe during the 1990s. At that time, the scientific knowledge of prions was too limited to determine its role in the development of spontaneous cases of BSE and their subsequent impact on human health. By 2011, however, several new prion strains, naturally occurring (19, 20) or artificially generated (10), have been described, indicating that their number has increased and that a species should be considered resistant to disease only after careful consideration.

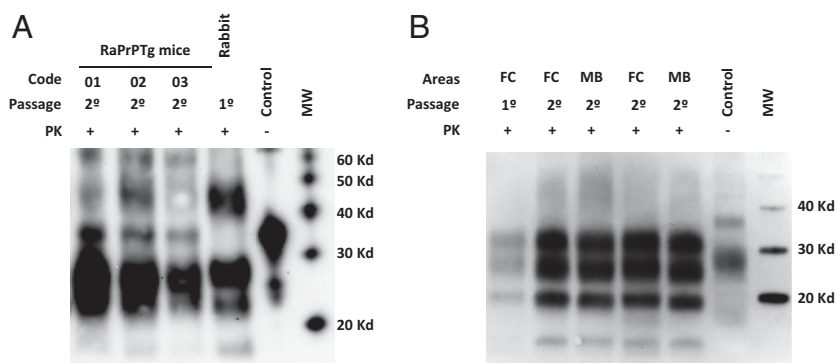
The degree of resistance and susceptibility to prion disease(s) differs within species. Animals can be both extremely susceptible to the majority of prion diseases or strains yet remain resistant to others on first passage (21). Therefore, it would be unwise to assume that a species generally resistant to TSEs would represent a minor risk to human health, as new TSEs and strains are continually being detected.

It is notoriously difficult to predict how a new TSE or strain will behave in different species, so great caution must be exercised when determining the transmissibility of prions between species. To evaluate the potential risk of transmission, every tool in the prion toolkit is essential, including artificial methods, such as the use of transgenic animals, or secondary in vivo transmission, which can exaggerate the possibility of infection (2, 22). Even these extreme measures, which probably do not reflect the normal mechanisms of infection, have to be considered to avoid future epidemics similar to that observed with the new variant Creutzfeldt-Jakob disease (vCJD). For this reason, we selected PMCA with its associated advantages, including the ability to overcome the transmission barrier (8), as the preferred tool to evaluate the absolute susceptibility of rabbits to TSEs.

Using only normal rabbit brain homogenate as a substrate for PMCA, PrP^C was efficiently converted to rabbit PrP^{res} when seeded with strains originating from four different species (Table 1), and at least three biochemically distinguishable rabbit PrP^{res} strains were obtained (Fig. 1). This suggests that rabbit PrP^C is more readily misfolded than originally thought. However, a prion disease is more than a simple misfolding process, and other factors are probably required for successful disease progression in vivo.

To address this point, we inoculated three groups of rabbits, two groups with ME7 based inocula (murine and leporine) and a further group with the de novo rabbit PMCA derived PrP^{res}. Selection of these particular strains was carried out on the basis that ME7 was stable and well characterized and the de novo

Fig. 5. Electrophoretic patterns of rabbit and rabbit PrP over-expressing transgenic (raPrPTg) mouse adapted de novo RaTgPrP^{Sc} strains. (A) Brain homogenate from de novo RaPrP^{Sc}-inoculated rabbit (lanes 1 and 5) was compared biochemically with three brain homogenates from de novo RaPrP^{Sc}-inoculated raPrPTg mice (coded as 01, 02, and 03). Both types of samples [rabbit (first passage) and raPrPTg (second passage)-derived prion strains] were indistinguishable according to the electrophoretic migration pattern and glycosylation profile, suggesting that de novo RaPrP^{Sc} strain was stable after its transmission in a rabbit PrP transgenic model. (B) Brain homogenate from de novo RaPrP^{Sc}-inoculated rabbit (first passage, lane 1) was compared biochemically with two brain homogenates from de novo RaPrP^{Sc}-inoculated rabbits (second passage, lanes 2–5). Two different areas [frontal cortex (FC) and midbrain (MD)] from two animals (477 dpi, lanes 2–3; 540 dpi, lanes 4–5) were analyzed. Both types of samples [rabbit (first passage) and rabbit (second passage)-derived prion strains] were indistinguishable according to the electrophoretic migration pattern and glycosylation profile, suggesting that de novo RaPrP^{Sc} strain was stable after its transmission in rabbits. Samples were digested with 100 μ g/mL of proteinase K and were analyzed by Western blot using monoclonal antibodies L42 (Left) and SAF84 (Right). Control, normal rabbit brain homogenate.



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strain was an unknown entity. The number of strains tested and rabbits challenged were limited because of the necessity of housing these animals in containment level 3 facilities for protracted periods of time that maximized the possibility of transmission and development of disease.

Although rabbits were inoculated with prions derived from the same species, a 25-mo period was necessary to observe the standard prion disease. The three animals that died without clinical signs of TSE were all negative by IHC and WB, although the first of these deaths was in a rabbit challenged with ME7 RaPrP^{Sc}, which had PrP^{Sc} levels in the brain detectable only by PMCA. This result indicates that even though the animal died of a non-TSE-related disease, it might have been at an early preclinical stage of prion disease. However, we cannot rule out the possibility that the limited PrP^{Sc} level in the brain sample was due to residual inoculum, as shown previously in PrP knock-out mice (23). To date, the rabbits inoculated with mouse ME7 remain healthy after more than 43 mo, which is in agreement with the experiments performed approximately 35 y ago by Barlow and Rennie (17).

In summary, after 3 y postchallenge with three different rabbit-derived inocula, we have obtained one positive clinical case, one possible preclinical case, two intercurrent deaths, and six animals that have remained healthy. Although the incubation periods do not directly correlate with the degree of susceptibility, these data might indicate that rabbits are poorly susceptible to prion infection. Although the rabbits used in this study were not inbred, they all had identical full-length PrP sequences and, to date, no difference has been detected in the ORF PrP sequence in any other published rabbit PrP sequence placed in GenBank. To further investigate this, two types of second passage experiment were performed; three raPrP^{Tg} mice and 10 rabbits were all intracerebrally inoculated using brain homogenate from the clinically affected rabbit. In contrast to 100% of the de novo RaPrP^{Sc}-inoculated transgenic mice having succumbed to a standard clinical prion disease and thereby demonstrating a high rate of transmissibility in vivo, two of 10 rabbits developed a TSE (477 and 540 dpi, respectively) to date. A plausible explanation for the evident differences between these two transmission studies would be the high level of rabbit PrP^C expression (4- to 6-fold) in the murine model. In addition, it is well known that even if overexpression does not increase susceptibility, it can significantly reduce the incubation time of disease (2). However, the two positive TSE cases in the second rabbit passage, even though 8 rabbits remained clinically normal at 560 dpi, have led us to conclude that rabbits can no longer be considered a prion-resistant species. The long incubation times, even after a second passage, might be due to the presence of some unknown, and probably rare, susceptibility factor in rabbits, which may also be present, for example, in equids and canids.

To critically evaluate this risk, several experiments are currently underway to characterize this new prion disease in rabbits and other species to examine its ability to cross the species barrier. In addition, supplementary experiments have been initiated in rabbits and also in transgenic mice that overexpress rabbit PrP^C, to evaluate their susceptibilities to other important prion diseases including CWD and BSE. There are several factors that any potential new TSE epidemic would require: (i) the new prion should be efficiently transmitted through the homologous species; (ii) animals should be edible by humans and should be slaughtered at an age at which the disease has developed, thereby increasing the chance that prions have replicated (especially for those prions that require long incubation times); and (iii) the meat and bone meal should be recycled and fed to new members of the same species. In the light of these data and taking into account the previous three factors, it is unlikely there will be an outbreak of "mad rabbit disease," and consumers of rabbit meat face much less of a risk than consumers of cattle or sheep products.

Methods

Inocula Preparation for in Vitro Prion Replication Studies. Brain homogenates (10⁻¹ in PBS) were prepared using scrapie mouse adapted prion strains (ME7,

RML, 22F, 79A, 139A, and 22L), scrapie sheep prion strains (5SBPB/1 and CH1641), and deer CWD and cattle BSE prion strains from the brains of clinically positive animals.

Generation of RaPrP^{res} by saPMCA. The in vitro prion replication, including the PrP^{res} detection of amplified samples, was performed following the basic conditions described previously (5). Briefly, 5- μ L aliquots of 10% (wt/vol) brain homogenate from animals infected by each strain were diluted into 50 μ L of 10% normal rabbit brain homogenate and loaded onto 0.2-mL PCR tubes. Tubes were positioned on an adaptor placed on the plate holder of a microsonicator (Misonix Model 4000, Farmingdale, NY) and programmed to perform cycles of 30-min incubation at 38 °C followed by a 20-s pulse of sonication set at potency of 80. Samples were incubated without shaking in the water bath of the sonicator. Serial rounds of PMCA consisted of 48 h of standard PMCA followed by serial in vitro 1:10 passages in fresh rabbit brain substrate. The detailed protocol for PMCA, including reagents, solutions, and troubleshooting, has been published elsewhere (4).

Biochemical Characterization of in Vitro- and in Vivo-Generated Prion Strains.

The standard procedure to digest PrP^{Sc} was performed following the basic conditions described previously (5). To study the profile of PK sensitivity for the in vivo-generated RaPrP^{res}, samples were incubated for 60 min at 42 °C with different concentrations of PK ranging from 0 to 16,000 μ g/mL. Digestion was stopped by adding electrophoresis loading buffer, and the samples were analyzed by Western blotting.

Stability Studies with Denaturing Agents. The RaPrP^{Sc} sample produced in vivo was treated with different concentrations (1.5–4 mol/L) of guanidine hydrochloride (GdHCl) for 2 h before being centrifuged at 25,000 \times g for 1 h at 4 °C. ME7 and RML prion strains were used as reference. The pellets were resuspended and treated with PK (50–100 μ g/mL). PrP^{Sc} signal was detected using Western blotting.

Preparation of ME7 and RaPrP^{Sc} Inocula. Brain homogenate, diluted 10⁻¹ in sterile 0.9% saline solution, was prepared from the brains of mice challenged intracerebrally with ME7 that had developed clinical signs of TSE. ME7 and de novo RaPrP^{Sc} samples generated in vitro during round 20 of saPMCA were diluted 10⁻¹ in sterile PBS.

Rabbit DNA Sequencing. Chromosomal DNA was extracted from rabbit tissue using DNeasy blood and tissue kit (Qiagen) for use as a template for PCR using HotStarTaq DNA polymerase (Qiagen) with the forward primer RaPrP/F (5'-ATGGCGCACTCGGCTAC-3') and the reverse primer RaPrP/R (5'-TCATCC-CAGCATCAGGAAG-3'). Primers were designed according to the published sequence of the *Oryctolagus cuniculus prnp* gene (GenBank accession no. U28334). PCR was performed in a Hybaid MBS 0.2G Thermal Cycler and included 5 min of initial denaturation at 95 °C followed by 35 cycles of 95 °C for 20 s, 56 °C for 45 s, and 72 °C for 1 min. The amplified product from each reaction was purified with a kit (Qiagen) and sequenced in both directions by Eurofins MWG Operon using the same primers as above. All sequences were compared at the nucleotide level and translated to provide the corresponding amino acid sequence. DNA and amino acid sequences were compared using SeqMan software (DNASTAR).

Inoculation. Ten 3-mo-old New Zealand White male rabbits (Harlan) were subjected to gaseous anesthetic (isoflurane; Abbott Laboratories). The left side of the scalp was shaved and prepared as for aseptic surgery, and 0.1 mL of prewarmed (37 °C) inoculum was injected into the left cerebral hemisphere using a 23-gauge hypodermic needle with a collar applied to restrict penetration to ~7 mm.

Transgenic Mice. A raPrP^{Tg} mouse line was generated and characterized as previously described (24). These mice express ~4- to 6-fold the normal rabbit PrP protein under the control of the murine PrP promoter in a murine PrP^{0/0} background.

Cell Culture Studies. Mouse neuronal cells in which PrP expression had been ablated (CF10 cells) (18) were transduced with a retrovirus encoding rabbit PrP^C, mouse PrP^C containing the epitope to the mouse monoclonal antibody 3F4 (Mo3F4PrP^S), or vector alone (pSFF) using previously described techniques (18). For mouse scrapie infection, cells were exposed to 200 μ L of a 1% brain homogenate derived from a 22L-infected, transgenic mouse displaying clinical disease. These transgenic mice express mouse PrP^C without the glycosylphosphatidylinositol membrane anchor. After 4 h at 37 °C, an additional

1 mL of medium was added, and the culture was incubated for an additional 3 d at 37 °C.

When the cells were confluent, the medium was removed and the cells in one well were trypsinized and replated into a single well of a six-well plate (passage 1). Thereafter, cells were trypsinized and passaged in six-well plates at a 1:10 dilution. The cells from the duplicate well were trypsinized, lysed in 500 μ L of lysis buffer (0.5% Triton X-100, 0.5% sodium deoxycholate, 150 mmol/L NaCl, 50 mmol/L Tris-HCl, 5 mmol/L EDTA), centrifuged at $16,000 \times g$ for 5 min, and the resultant lysate supernatant stored at -20 °C for PrP^{Sc} analysis (passage 0). For detection of PrP^{Sc} at different cell passes, 500 μ L of cell lysate, derived as described above, was digested with 20 μ g/mL of proteinase K (PK; Roche) for 1 h at 37 °C. Following the addition of 1 mmol/L Pefabloc (Roche), lysates were centrifuged at $200,000 \times g$ for 1 h at 4 °C. The resultant pellet was sonicated into 15 μ L of sample buffer (5% SDS, 3 mmol/L EDTA, 2% β -mercaptoethanol, 5% glycerol, 0.02% bromophenol blue, 63 mmol/L Tris-HCl), and boiled for 5 min, and the entire sample was loaded onto 16% Tris-glycine precast gels (Invitrogen). Following transfer to PVDF membrane (Millipore), rabbit PrP was detected with the mouse monoclonal antibody L42 (1:3,000 dilution, R-Biopharm AG) and Mo3F4 PrP with the mouse monoclonal antibody 3F4 (1:25,000 dilution, mouse ascitic fluid). Blots were developed using the enhanced chemiluminescence system according to the instructions of the manufacturer (GE Healthcare).

Transmission Studies Using Rabbit PrP Transgenic Mice. Groups of three and five mice (8 wk old) were housed following the guidelines of the Code for Methods and Welfare Considerations in Behavioral Research on Animals (Directive 86/609EC). Mice were inoculated in the right parietal lobe using a disposable 25-gauge hypodermic syringe. A 20- μ L quantity of 1% de novo raPrP^{Sc} inoculated brain or healthy brain homogenates was delivered to each animal. The neurological status of the inoculated mice was assessed twice weekly. The presence of three signs of neurological dysfunction of 10 established diagnostic criteria was needed to score a mouse as positive for prion disease. When progression of the disease was evident, the animals were killed for ethical reasons, and their brains were harvested for subsequent biochemical and histological analysis.

Histology. Brain was collected postmortem from rabbits and sliced sagittally down the middle, with one half stored frozen (-80 °C) for use in biochemical studies and the preparation of inocula for future experiments, and the other half fixed in 10% buffered formalin. After 1 wk in fixative selected areas of brain (frontal cortex, midbrain, cerebellum, and obex) were processed using standard protocols (dehydrated through graded alcohols, embedded in paraffin wax, sectioned in 4- μ m sections, mounted on glass microscope slides) and stained with hematoxylin and eosin.

Immunohistochemistry. Additional tissue sections were cut and mounted onto Superfrost Plus microscope slides (Menzel) and dried overnight at 37 °C. The method for labeling of glial fibrillary acidic protein (GFAP) and PrP^{Sc} was identical, except for the primary antibody used and the omission of formic acid treatment for GFAP. Sections were rehydrated through graded alcohols to water and were then immersed in 98% formic acid for 10 min at room

temperature (RT). Antigen retrieval consisted of autoclaving the sections in 0.01 mol/L citrate buffer, pH6, at 121 °C for 10 min. Endogenous peroxidase activity was blocked by placing sections in 3% hydrogen peroxide in methanol (vol/vol) for 20 min at RT. Subsequently slides were placed in the Sequenza slide rack system (ThermoFisher Scientific) for the rest of the method and 0.3 mol/L PBS containing 0.05% Tween 20 was used for all dilutions of antibodies and washes. Nonspecific binding was inhibited by applying 25% normal goat serum (Vector Laboratories) for 30 min at RT. Sections were incubated overnight at 4 °C with either mouse anti-prion protein antibody 2G11 (SPiBio, Montigny, Le Bretonneux, France) at 0.5 μ g/mL or mouse anti-GFAP antibody [6F2] (clone ab86144; Abcam) prediluted by the manufacturer. Visualization was performed via goat anti-mouse IgG antibody with a peroxidase labeled polymer (EnVision+ System-HRP; DakoCytomation) that was applied for 30 min at RT followed by DAB chromogen (Dako) for 8 min before being washed in water, counterstained with hematoxylin, blued up with Scot's tap water substitute, dehydrated, cleared, and mounted.

Pet-Blot. Further tissue sections (4- μ m) were cut and mounted onto 0.45- μ m nitrocellulose membrane (Sigma, Dorset, United Kingdom) and dried for 48 hours at 60 °C. The nitrocellulose membrane-mounted section was de-paraffinized with xylene and then rehydrated using isopropanol gradients. All washes were carried out using TBS with Tween 20 (TBST) buffer pH 7.6 (0.1 mol/L Tris-HCl, 0.1 mol/L di-NaCl, 0.05% Tween 20) unless stated. Sections were subjected to 50 μ g/mL PK at 55 °C overnight. PK digestion was halted using 1 mmol/L PEFA block (Pierce Biotechnology) for 15 min at RT. Further denaturation of the protein was carried out using 4 mol/L guanidine thiocyanate for 15 min. Sections were then washed three times before addition of 2% casein for 60 min to block nonspecific binding. Subsequent to this, sections were incubated with antibody SAF 84 (SPiBio, at 2 μ g/mL in blocking solution) for 90 min. After washing, sections were incubated with rabbit anti-mouse IgG:alkaline phosphatase antibody (Pierce Biotechnology), diluted 1:200, for 90 min. Sections were washed twice for 5 min in TMN solution (0.1 mol/L Tris/HCl, 0.1 mol/L di-NaCl, 0.005 mol/L di-MgCl₂ at pH9) before incubation with the chromagen nitro-blue tetrazolium chloride/5-bromo-4-chloro-3'-indolylphosphate p-toluidine salt (NBT/BCIP; Perbio Science) for 1–5 min, until labeling of the positive control sections was clear. Sections were then washed with an EDTA buffer (0.1 mol/L Tris-HCl, 0.1 mol/L di-NaCl, 0.02 mol/L EDTA) twice for 2 min and air dried at RT.

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