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Naturally prion resistant mammals

A utopia?

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Each known abnormal prion protein (PrP^{Sc}) is considered to have a specific range and therefore the ability to infect some species and not others. Consequently, some species have been assumed to be prion disease resistant as no successful natural or experimental challenge infections have been reported. This assumption suggested that, independent of the virulence of the PrP^{Sc} strain, normal prion protein (PrP^{C}) from these "resistant" species could not be induced to misfold. Numerous in vitro and in vivo studies trying to corroborate the unique properties of PrP^{Sc} have been undertaken. The results presented in the article "Rabbits are not resistant to prion infection" demonstrated that normal rabbit PrP^{C}, which was considered to be resistant to prion disease, can be misfolded to PrP^{Sc} and subsequently used to infect and transmit a standard prion disease to leporids. Using the concept of species resistance to prion disease, we will discuss the mistake of attributing species specific prion disease resistance based purely on the absence of natural cases and incomplete in vivo challenges. The BSE epidemic was partially due to an underestimation of species barriers. To repeat this error would be unacceptable, especially if present knowledge and techniques can show a theoretical risk. Now that the myth of prion disease resistance has been refuted it is time to re-evaluate, using the new powerful tools available in modern prion laboratories, whether any other species could be at risk.

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Introduction

The first notion of, what retrospectively became known as a transmissible spongiform encephalopathy (TSE). emerged around 1730 in France with the description of a rare disease in sheep called "tremblante."[15] Over time, observations of and investigations into natural TSE cases resulted in an increasing list of TSEs and species susceptible to this family of diseases. However, species which were apparently resistant to clinical TSE occurred, even when they lived alongside those that were susceptible and showing clinical disease, for example rabbits and sheep respectively. Therefore, study of these apparently resistant species was thought to be critical to understanding the species susceptibility to TSEs and the mechanism of prion disease resistance. Before prions were defined as infectious proteins and found to be responsible for TSEs, Gibbs and Gajdusek (1973) made many unsuccessful attempts to infect guinea pigs and rabbits with the ME7 prion strain which is highly transmissible to several other prion susceptible species including sheep, mice, hamsters and field voles (*Microtus agrestis*). In addition, the challenge of suspected prion resistant species with other known prion strains, such as Creutzfeldt-Jakob disease (CJD) or kuru, also failed to produce clinical disease.[4,20] However this may be explained by disease incubation periods being longer than the lifespan of the challenged animal species.[16]

Although the theory of prion strain dependent virulence seemed to be more logical than the existence of species that were resistant due to polymorphism in the *prnp* or other genes, new data arose in Europe at the time of the bovine spongiform encephalopathy (BSE) crisis which questioned this. Reports of BSE crossing the species barrier in some exotic animals but not others within zoological collections raised the concept of the "prion resistant species" again. How was it possible that some species subjected to the same infectious agent and with a similar lifespan as the susceptible species did not develop disease? For example, equids, canids or rabbits were all fed BSE contaminated food.[23,30] These observations revived the idea of the existence of prion resistant species and furthermore highlighted leporids as a useful experimental target to understand the mechanism of prion disease resistance.

Prion Resistant Species: A Difficult Concept To Prove

Several studies focusing on the structural stability of rabbit PrP^{C} have attempted to decipher the mechanisms of disease resistance in leporids and this has yielded new and interesting data that appears to be consistent with the behavior of TSE-challenged leporids.[19,33,35,36] Nevertheless, to assume that prion resistance occurs purely due to certain PrP amino acid residue sequences could be unfortunate. For example, the alleged prion resistance of sheep carrying the ARR PrP polymorphism[26] has resulted in the adoption of scrapie resistant breeding programs throughout Europe. However, it was predicted by both in vitro and transgenic mouse tools[6,9] that this PrP polymorphism would not provide complete resistance and this was demonstrated by reports of TSE-infected ARR sheep which were characterized by long incubation periods.[22] The large number of sheep that are exposed to the highly adaptable scrapie agent suggests it is unlikely that a resistant polymorphic PrP isoform can emerge naturally.

Conversely, inappropriate experimental studies performed using limited tools could generate incorrect assumptions. Thus, if transgenic mice based on knock-in technology expressing a single copy of human PrP are used to evaluate the transmission barrier of BSE, we would have to conclude that human PrP is resistant to infection with BSE[8,26] but this is not correct as proved by the cases of variant CJD (vCJD) that have been reported.[33] It was necessary to develop transgenic mice overexpressing human PrP to demonstrate, in murine experimental studies, the ability of BSE to infect humans.[3,7,24] This example is a reminder that natural evolution has much larger cohorts than those available in any laboratory, thereby greatly increasing any theoretical chance an event may occur to the point where it occurs naturally. Therefore, unsuccessful transmission experiments must be evaluated carefully before any conclusions are drawn and subsequent policies introduced.

Experiences in the field of TSE research emphasize the importance of using all available resources to predict the resistance or susceptibility of a particular species. This should be independent of any anticipated probability that an infection may take place as natural evolution and selection is introducing changes continuously or a consequence of human manipulation.

The protein misfolding cyclic amplification (PMCA)[13] technique and transgenic animals overexpressing PrP of different species have become the best tools for evaluating and characterizing transmissibility with respect to studying species barriers, including those that have not been crossed naturally to date.[5,11,18,21]

The Birth of a Rabbit Prion

As shown in the publication "Rabbits are not resistant to prion infection,"[14] each tool in the prion toolkit was used to challenge the hypothesis of prion resistance in rabbits. First, PMCA allowed serial passages to be performed with many of the available prion strains emulating years of infection pressure in rabbits in a short period of time. Second, in vivo transmissions performed in rabbits and also transgenic mice overexpressing rabbit PrP showed efficient transmission of the new rabbit derived prion.

All the prion strains examined amplified by PMCA readily in rabbit brain homogenate when compared with other species considered to be prion resistant, such as horses and dogs, in which replication was impeded (unpublished data). Three biochemically different migration patterns were observed on western blotting suggesting that rabbit PrP^{C} is more able to be readily misfolded than originally considered. This was supported further by the generation by PMCA of a spontaneous PK resistant rabbit PrP ($RaPrP^{res}$) from unseeded normal rabbit brain homogenate. Although these in vitro data suggested that $RaPrP^{res}$ might have been infectious in vivo, a prion disease is more than a simple misfolding process and other factors are required for successful disease progression in vivo as was demonstrated in the original publication. At 766 days post inoculation (dpi), by the intracerebral route, one of the animals challenged with the $RaPrP^{res}$ derived from the unseeded rabbit brain homogenate developed clinical signs and detectable PrP^{Sc} in many areas of the brain. Despite this initial promising in vivo result, 4 years after inoculation none of the remaining animals inoculated with the unseeded strain, the rabbit amplified ME7 prion strain or with the original ME7 showed any sign of disease or PrP^{Sc} accumulation in the brains suggesting low infectivity during the primary transmission.

Our challenge with classical murine ME7 did not result in transmission by 48 months, similar to the experiments performed in rabbits in the 1960s where none of the ME7 inoculated animals developed clinical signs after 52 months.[4] When we challenged rabbits with ME7 which had been previously subjected to PMCA in normal rabbit brain homogenate, one of the animals died suddenly without any promontory clinical signs after 818 dpi. The brain from this animal was negative for abnormal PrP by IHC and western blot. However, after further amplification by PMCA, this sample became positive by western blot.

The low attack rate of the unseeded in vitro prion strain (only a single animal developing clinical signs typical of a TSE) might be explained through the generation of a strain which converted PrP^{C} to PrP^{Sc} very slowly thereby yielding a low attack rate in the primary infection or as a consequence of an in vitro/in vivo transmission

barrier which would require further adaptation in a subsequent secondary passage within the same host.

Rabbit Prions are Infectious and Transmissible

In order to explore if the new rabbit prion strain required further adaptation and to characterize its transmissibility in the same species, a second passage was performed in five rabbits using brain homogenate from the single positive animal which developed clinical disease. This resulted in a 100% attack rate and a mean incubation time of 569.4 ± 12.4 dpi demonstrating the ability of this rabbit prion strain to infect other rabbits. Further characterization of this transmissibility is being performed at present using mice overexpressing rabbit PrP.

Despite the low efficiency of primary experimental infection it is still unclear why no cases of prion disease have been reported in populations of rabbits worldwide which commonly cohabit with potential carriers of TSEs such as sheep and cattle in Europe, and deer in the United States and Canada. To address this question, a panel of prion strains has been inoculated into transgenic mice overexpressing rabbit PrP (experiments ongoing). We believe that the results from this experiment will reaffirm the concept that impaired transmissibility should not be considered as being caused by species barriers but as prion strain barriers. Several examples support this idea; while wild type mouse models are considered highly susceptible to prions, the same model is completely resistant to the prion strain causing chronic wasting disease (CWD) in cervids.[30] In order to infect mice with CWD it is necessary to overexpress mouse PrP through the generation of transgenic models such as tga20 mice.[28] This suggests that the susceptibility of mice to prion diseases is more dependent on the strain than on the species per se. A similar phenomenon is also observed in bank voles which are highly susceptible to most prion strains, but not easily infected with BSE in first passage and furthermore, a second passage of over 500 dpi was required to generate a bank vole adapted BSE strain.[1]

In terms of unconventional experimental species, we could highlight this phenomenon in several species such as pigs and cats. Pigs were considered prion resistant as no natural cases have been observed despite a large population and being fed intensely with feedstuffs containing animal derived protein. However, it has been demonstrated that BSE is able to infect pigs albeit with low efficiency, while infection using scrapie strains in transgenic mice overexpressing porcine PrP was completely unsuccessful.[12,17] Conversely, cats were considered susceptible as experimental challenge with CJD was successful[20] and subsequently natural cases of infection with BSE have been reported.[2] However, when cats were challenged with CWD it was necessary to wait 40–43 months for the first passage and nearly 2 years for the second passage in order to confirm their susceptibility to this strain [Nalls A, et al. (private comunication)].

Therefore, in order to successfully cross a transmission barrier several aspects should be considered. First, increase the chance of host PrP misfolding with the given prion strain by in vitro techniques and/or by challenging transgenic mice overexpressing PrP. Second, consideration should be given to factors other than PrP which may play a key role such as the brain size/complexity and the host lifespan. This may require extending the duration of the experiment as close as possible to the natural lifespan of the challenged species and/or performing serial in vivo inoculations. Unfortunately, previous reports of expected incubation times inferred from other species, although closely related, are not representative.

Evaluating the Risk of Rabbit Prions

Evaluation of cross species transmission is fundamental to establishing risk and vital if the susceptible species to prion disease is in the human food chain. These transmission experiments are being performed at present using the new rabbit prion strain in both mouse and bank vole models as these laboratory species are highly characterized and regularly used for strain typing. Likewise, transgenic mice overexpressing human PrP have also been challenged to evaluate possible risks related to human consumption of rabbits and the results eagerly awaited. The presumption of possible transmission to humans may sound exaggerated as historically no natural cases have been reported and we have shown that despite rabbits being susceptible, they do not appear to be highly vulnerable to prion infections. As discussed, it is possible that our challenge studies were not performed using the most adequate prion strain and it could be that other prion strains that we have now developed in rabbit brain homogenate from BSE and scrapie may result in more efficient transmission in this host. However, we have shown that rabbits are susceptible to prion disease[14] and the possibility that leporids could sustain a slow prion disease transmissible to other cohabiting species or even enter into the human food chain cannot be ruled out. For these reasons, it is essential to evaluate all the pathobiological properties of prion strains recovered after their adaptation to a new species as it is well known that some prion strains change their virulence and/or ability to infect certain species after they are adapted to intermediate species, e.g., increased virulence of BSE after it is passaged through sheep.[24] Since the origin of the new rabbit prion strain generated in vitro was spontaneous, we cannot predict the virulence and its species spectrum of susceptibility. With respect to this, rabbit in vitro amplified BSE is able to propagate a TSE in transgenic mice overexpressing bovine PrP and, alarmingly, the recovered rabbit adapted BSE strain retains all the characteristic BSE features including the ability to infect transgenic mice overexpressing human PrP (unpublished data).

Some reports have shown an atypical biochemical behavior of recombinant rabbit PrP when compared with recombinant PrP of species already known to be susceptible to prion diseases.[19,32,34,35] Despite data suggesting rabbit PrP showed a resistance to misfolding, these studies did not determine which specific amino acid residues may be responsible.[32,34] Studies to address this are ongoing in our laboratory to assess each independent amino acid that could be implicated in the misfolding process and that may have been responsible for categorising leporids as a prion resistant

species. Previous studies, performed with well characterized prion strains and hosts such as scrapie and mice respectively, identified some important amino acid residues involved in the species barriers between rabbits, mice and hamsters.[31] However, these studies need to be performed with every prion disease strain as each amino acid residue may have a different role depending on the specific misfolding occurring in each prion protein. Such studies would confirm the importance of the hypothesis that there are no species barriers but strain barriers. In most well-studied species, the *prnp* gene is highly polymorphic suggesting a similar situation may be found in rabbits. The identification of any PrP polymorphisms present in different species of leporids or between breeds of rabbit is required to determine their implication in susceptibility/resistance to prion diseases, especially because all the rabbits used in our study had identical PrP sequences.

Concluding Remarks

Rabbits were historically defined as a prion disease-resistant species, as no natural cases were reported and laboratory challenges had been unsuccessful. In vitro amplification tools have allowed the generation of a new prion strain able to infect rabbits and to be transmitted very efficiently (100% attack rate on second passage).

Now we have proven susceptibility of rabbits to prion disease, it is vital to re-examine the resistance or susceptibility of apparently prion disease resistant mammals to anticipate the plausibility of new TSEs occurring. This would aid management of future outbreaks by enabling the implementation of control policies based on rigorous scientific data. In retrospect, the BSE crisis would have resulted in less damage if restrictions on feeding ruminant derived protein to ruminants had been enforced earlier and more rigorously. It is remarkable that current European regulation controls the feeding of farmed animals based on studies demonstrating the resistance/susceptibility of species to different prion strains due to their ability to transmit to humans. Our studies strongly support a complete ban on mammalian derived protein being fed to animals in the human food chain. In the light of our findings in rabbits[14] (and other unpublished data that prove that none of the purportedly resistant mammalian species including canids and equids), any animal-animal feeding should be carefully evaluated via risk assessments using data derived from state of the art techniques in prion research.

Prions and their remarkable propriety of adaptation could be compared with a highly mutagenic virus devoid of species limitation if given enough time and opportunity. Ten years ago the existence of atypical prions was not considered important yet now it is considered to be a common phenomenon affecting all species that are being studied sufficiently. Prion strains are a dynamic biological entity inclined to change and as a consequence of this, new strains with distinct virulence and spectra of susceptible hosts are "spontaneously" emerging[10,27] or adapting after recurrent passage through different hosts. Therefore, it would be unwise to consider any species, even those never reported as susceptible to prion infection, could not become a risk to human health.

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