Genetic prion disease: no role for the immune system in disease pathogenesis?

Yael Friedman-Levi†, Orli Binyamin†, Kati Frid, Haim Ovadia and Ruth Gabizon*

Department of Neurology, The Agnes Ginges Center for Human Neurogenetics, Hadassah University Hospital, Jerusalem, Israel

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Prion diseases, which can manifest by transmissible, sporadic or genetic etiologies, share several common features, such as a fatal neurodegenerative outcome and the aberrant accumulation of proteinase K (PK)-resistant PrP forms in the CNS. In infectious prion diseases, such as scrapie in mice, prions first replicate in immune organs, then invade the CNS via ascending peripheral tracts, finally causing death. Accelerated neuroinvasion and death occurs when activated prion-infected immune cells infiltrate into the CNS, as is the case for scrapie-infected mice induced for experimental autoimmune encephalomyelitis (EAE), a CNS inflammatory insult. To establish whether the immune system plays such a central role also in genetic prion diseases, we induced EAE in TgMHu2ME199K mice, a line mimicking for late onset genetic Creutzfeldt Jacob disease (gCJD), a human prion disease. We show here that EAE induction of TgMHu2ME199K mice neither accelerated nor aggravated prion disease manifestation. Concomitantly, we present evidence that PK-resistant PrP forms were absent from CNS immune infiltrates, and most surprisingly also from lymph nodes and spleens of TgMHu2ME199K mice at all ages and stages of disease. These results imply that the mechanism of genetic prion disease differs widely from that of the infectious presentation, and that the conversion of mutant PrPs into PK resistant forms occurs mostly/only in the CNS. If the absence of pathogenic PrP forms form immune organs is also true for gCJD patients, it may suggest their blood is devoid of prion infectivity.

INTRODUCTION

Prion diseases are a group of fatal, late onset neurodegenerative disorders, such as Creutzfeldt–Jakob disease (CJD) in humans and scrapie in sheep and in rodents (1). CJD can present at sporadic or infectious etiologies, but also by a dominant genetic trait, linked to pathogenic mutations in the PrP protein (2–4). The most prevalent gCJD form is the one linked to a glutamate for lysine substitution at codon 200 (E200K) of the PrP protein (5). E200K gCJD is especially common among Jews of Libyan origin (6,7), but has also been identified in other clusters around the world (8).

The mechanism leading to infectious prion disease manifestation is believed to follow the following pathway (9). First, and regardless of the site of entry, prions replicate in lymphoid organs, as shown by the fact that prion infectivity and the accumulation of proteinase K (PK)-resistant PrP forms, PrPSc (10,11), the only identified prion component, are initially detected in the spleens of the infected animals (12–15). Prions invade the nervous system mostly by transmigration of infected lymphoid cells as well as retrograde transport in ascending peripheral tracts (15–20). Once prions enter the brain, replication of infectivity and accumulation of PrPSc occurs faster than in the lymphoid system, subsequently leading to the death of the affected subject (21–23).

Under inflammatory conditions, PrPSc deposits can be detected in the sites of infiltration (24), as is the case for mastitis in scrapie-infected sheep, which results in deposition of PrPSc in the inflamed mammary glands (25). Moreover, in the presence of CNS inflammatory insults, such as experimental autoimmune encephalomyelitis (EAE), prion-infected mice presented both deposition of PrPSc in inflamed infiltrated areas and acceleration of the fatal disease, consistent with what can be considered as an accelerated prion neuroinvasion (26,27).

In this work, we asked whether activation of the immune system plays an accelerating role also in the pathogenesis of genetic prion disease. While the kinetics of disease progression in earlier stages as well as disease-related PrP accumulation

† Co first author.

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cannot be investigated in humans, we have shown in our TgMHu2ME199K mice, modeling for E200K CJD (28) that mutant PrP in brains converts into a PK-resistant form starting from 3 months of age, before clinical signs are apparent, and accumulates further concomitant with initiation and progressive aggravation of clinical signs in the mice (6–12 months old). Regrettably, features of the last stage of disease in humans, rapid deterioration and death, cannot be evaluated in mice owing to limitations of ethical permits. Therefore, while the actual presence of disease-related PrP forms in the brains of TgMHu2ME199K mice does not require a neuroinvasion process, an immune-mediated inflammatory insult carrying prion-infected infiltrates into the CNS may accelerate the onset of disease, as is the case for scrapie-infected mice (26,27). This possibility has important clinical implications for carriers of pathogenic PrP mutations, since it suggests that concurrent medical conditions may result in an earlier fatal disease.

To investigate this possibility, we induced EAE in TgMHu2ME199K mice and followed them first for EAE clinical signs and latter for prion symptoms. Subsequently, we looked for the accumulation of PK-resistant PrP forms in brains of the EAE-induced animals, as well as in immune infiltrates and spleens of different ages and disease scores.

We show here that, as opposed to the strong clinical effect of EAE induction on scrapie-infected mice, this CNS inflammatory insult had no effect on the time course of disease appearance and aggravation in TgMHu2ME199K mice or on the burden of disease at different stages. Most importantly, we could not detect both by pathological or by biochemical methods any disease-related PrP forms in the activated infiltrates or in the spleens of naïve or EAE-induced TgMHu2ME199K mice of different ages. This suggests that the initiation of the prion disease process in carriers of pathogenic PrP mutations may occur solely in the CNS, probably due to a metabolic trigger generated by the mutated PrP at oxidative stress/aging-related conditions. The absence of disease-related PrP forms form the immune system, if true for human genetic CJD patients, may suggest that their blood is devoid of prion infectivity.

RESULTS

EAE induction does not affect the clinical course of genetic prion disease

To investigate whether induction of an immune-mediated CNS inflammatory insult can change the kinetics and clinical outcome of genetic CJD, we induced EAE in 3-month-old TgMHu2ME199K mice on both a wt and a PrP ablated background, mimicking respectively for heterozygous and homozygous E200K CJD carriers/patients (see Fig. 1 for summary of published experiments and experimental design of present ones). This time point for chosen for EAE induction, because
it is still an asymptomatic point for the TgMHu2ME199K mice and yet disease-related PrP is already easily detected in brains, allowing for evaluation of disease aggravation due to induction. As controls, we induced EAE also in wt and in PrP ablated mice of the same murine background (C57B). The age of the mice used for the EAE induction (about 3 months old) was chosen so that while no clinical prion disease is apparent, PrPST, the prevalent disease-related PrP form in this model can already be detected at low levels in the mice brains (29). Since the genetic background can dramatically affect the severity of EAE clinical signs (30), all groups of mice used in these experiments, including TgMHu2ME199K/ko, TgMHu2ME199K/wt, wt C57B and PrP0/0, were littermates.

Acute EAE clinical signs (see Table A in Fig. 2) in all groups appeared first at 10 days post induction, increased for the next 10 days followed by a gradual recovery of the mice from neurological signs, as seen in Graph A in Figure 2. Scores over time were similar for all groups of mice, presenting no statistical difference (P > 0.05). These results are in contrast with previous publications in which recovery from the acute phase of EAE was more difficult for PrP0/0 mice when compared with wt mice (31,32). The variance between these and our results may relate to strain differences, which as stated above, are very important for the manifestation of clinical EAE. At the end of the acute phase (Fig. 2), mice in all groups recovered from most debilitating EAE symptoms but maintained low scores of chronic EAE for the duration of the experiments (see wt and PrP0/0 groups in Graph B of Fig. 2).

Next (150–250 days), we monitored the advance of genetic prion disease signs in the EAE-induced TgMHu2ME199K mice and compared them with those of naïve TgMHu2ME199K mice (see Table B in Fig. 2 for scores of genetic prion disease and Graph B for the kinetics of disease scores). Unlike the effect of EAE on scrapie-infected mice, our results shown no difference in the first appearance of clinical signs as well as in the rate of disease aggravation between TgMHu2ME199K mice induced or not induced for EAE. This was true for both TgMHu2ME199K/WT and TgMHu2ME199K/KO mice, indicating that the EAE insult did not change the outcome of disease regardless of the presence of a wt PrP allele, as is the case for heterozygous an homozygous TgMHu2ME199K mice in the absence of EAE (29).

Immune infiltrates in EAE-induced TgMHu2ME199K mice do not carry disease-related PrP

Spinal cords of mice infected with viable spleen cells from scrapie-infected mice induced for EAE cells presented marked subpial demyelination colocalized with immune infiltrates and with the accumulation of PrPSc in the white mater (26). To test whether immune infiltrates can carry disease-related PrP forms into the white matter of EAE-induced TgMHu2ME199K mice, we immunostained spinal cord sections of these animals with α-PrP pAb RTC (33). We have recently shown that in the TgMHu2ME199K line as well as in human E200K patients, disease-related PrP may appear in several forms, ranging from full-length PrP oligomers (‘classical PrPSc’) to truncated, soluble and PK-resistant forms denominated PrPST (29). These last forms are better recognized by C-terminally directed α-PrP antibodies such as RTC (29) or EP1802Y (Epitomics). Panel A in Figure 3 presents the α-PrP immunostaining of...
Figure 3. Immune infiltrates in EAE-induced TgMHu2ME199K mice do not carry disease-related PrP. Paraffin-embedded spinal cord sections of EAE-induced and naïve TgMHu2ME199K mice were immunostained for PrP with pAb RTC or with Luxol fast blue for myelin. (A–E), EAE-induced TgMHu2ME199K/wt mouse 197 days post induction: (A), α-PrP immunostaining (RTC and counterstain with hematoxylin) do not show aggregation in inflamed and demyelinated white matter areas as compared with gray matter areas (Original magnification × 40). Rectangle (B) is enlarged (original magnification × 100) showing infiltration of mononuclear immune cells and no PrP aggregation. Rectangle (C) is also enlarged (original magnification ×100) showing extensive intracellular PrP aggregation in spinal cord gray matter. (D), Serial section stained with Luxol fast blue/PAS showing demyelination in the same inflamed white matter area (original magnification ×40). Rectangle (E) is enlarged (original magnification ×100). (F) Spinal cord section of age matched TgMHu2ME199K/wt naïve mouse: α-PrP staining (RTC and counterstain with hematoxylin) shows excessive intracellular aggregation of PrP in gray matter as previously shown to be a feature of the genetic model (original magnification ×40). Rectangle (G) is enlarged (original magnification ×100). Rectangle (H) is also enlarged (original magnification ×100) showing no aggregation of intracellular PrP in non-inflamed white matter areas. The even distribution of brown signal represents mutant PrP<sup>Sc</sup> background.
spinal cords of EAE-TgMHu2ME199K mice, counterstained with hematoxylin, while Figure 3D presents a consecutive spinal cord section stained with Luxol fast blue for myelin. These figure shows extensive demyelination (insert Fig. 3E), concomitant with infiltration of immune cells (Fig. 3B), but not aggregated PrP forms in the infiltrated and demyelinated sections. Intracellular PrP aggregates were readily detected, however, only in the gray matter areas (Fig. 3A and C). A light smeared background PrP staining could be observed in all parts of the section, maybe representing residual mutant PrP forms. Similar results for PrP immunostaining were obtained for non-EAE-TgMHu2ME199K spinal cords (Fig. 3F and H), in the absence of demyelination and infiltration. These pathological data suggest that EAE activated immune cells in TgMHu2ME199K mice did not carry aggregated PrP forms when infiltrating the CNS, as opposed to those of EAE-induced scrapie-infected immune cells, consistent with the absence of an EAE-related clinical effect.

Lymphoid organs of MHu2ME199K mice do not accumulate disease-related PrP

In view of the previous results, we next looked for disease-related PrP forms in immune organs of TgMHu2ME199K mice at different ages. Indeed, in an array of prion-infected animals, the first place for PrPSc to accumulate is the spleen, lymph nodes or even tonsils, regardless of the great difficulty to detect PrPSc in blood. This is also true for infectious forms of prion disease in humans such as vCJD. However, the accumulation of PK resistant PrP forms in immune organs of genetic prion diseases was never reported.

Figure 4A presents the results of immunoblotting with α-PrP pAb RTC of spleen samples from TgMHu2ME199K, scrapie-infected and wt mice. As a positive PK-resistant sample, a homogenate from a TgMHu2ME199K brain was also included in the study. All samples were first fractionated as in the cartoon, a protocol designed to enrich PrPST in TgMHu2ME199K brains. The figure shows that while in the absence of PK digestion, PrP in the brains of the TgMHu2ME199K mice is present in both supernatants and in pellet3, PK-resistant PrP in the form of PrPST is present mostly in sup 1, as we recently reported. Contrarily, no PK-resistant or pelletable PrP was detected in the spleens of TgMHu2ME199K mice form different ages, such as asymptomatic 3.5-month-old, just-diagnosed 5-month-old and very sick 13-month-old, as was the case for the samples from wt mice. As expected, spleens from prion-infected mice demonstrate classical PrPSc in the pellet. We conclude that, in the absence of an inflammatory insult, no disease-related PrP forms were detected in the spleens of TgMHu2ME199K mice at different ages or disease conditions.

We next tested whether induction of EAE in TgMHu2ME199K mice affected the expression of PrP and its levels of
PK resistance in both spleen and brains. Figure 4B shows no significant difference between the levels of PrP in both organs following the induction, both before and after PK digestion. It also shows that, consistent with the clinical data, EAE induction did not generate PK-resistant PrP in the spleens of TgMHu2-ME199K mice. Finally, we also looked at the levels and PK resistance of PrP in the lymph nodes of wt, TgMHu2ME199K/ko, TgMHu2ME199K/wt and scrapie-infected mice. We found (Fig. 4C) that while the initial levels of PrPSc are lower in wt when compared with TgMHu2ME199K mice, probably because of overexpression of the mutant PrP in this transgenic line (28), PK-resistant PrP in the forms of PrP Sc was detected only in the lymph nodes of scrapie-infected mice, demonstrating again the absence of disease-related PrP in immune organs of TgMHu2ME199K mice. These results are consistent with the possibility that mutant PrP as expressed in immune organs cannot convert into PK-resistant PrP forms, thereby may not participate in disease initiation and further propagation.

DISCUSSION

We have shown here that induction of EAE to TgMHu2ME199K mice, a transgenic line mimicking for genetic CJD linked to the E200K mutation (37), did not accelerate disease onset, as opposed to the robust clinical effect that this inflammatory insult exerted on scrapie-infected mice (26,27). Concomitantly, we demonstrated that white matter spinal cords in EAE-induced TgMHu2ME199K mice did not present disease-related PrP forms, again in contrast to EAE-induced scrapie-infected mice. Most importantly, no disease-related PrP form, such as PrPSc or PrPST (29), were detected in spleens or lymph nodes from TgMHu2ME199K mice of different ages and disease status, indicating that in this genetic disease, the immune system is not a ‘station’ for prion replication in its way to the CNS (see Fig. 5 for summary of results). These results differ strongly from those observed for infectious prion diseases, where the accumulation of PrPSc in immune organs is considered the hallmark of disease initiation and progression (20,38).

Consistent with mice models of infectious prion disease, PrPSc was readily detected in spleens or tonsils of most transmissible CJD forms such as vCJD (39). However, PrPSc was identified only in the lymphoreticular tissues of some sporadic CJD cases (40,41). In addition, the levels of PrPSc present in the lymphoid tissues of a primate model of sCJD were significantly lower when compared with those in the same primates infected with vCJD, arguing for a non-critical involvement of the immune system in sporadic CJD pathogenesis (42). To the best of our knowledge, PrPSc was never reported in the immune system of gCJD patients (43). This indicates a wide mechanistic difference between transmissible and genetic prion diseases, in which neuroinvasion following prion replication in the immune system is not a feature for disease pathogenesis. Sporadic CJD cases may each have individual mechanisms in which the immune system may or not be involved.

E200K CJD is the most prevalent genetic human prion disease CJD form, and in addition presents the greatest clinical similarity to sporadic CJD (6). Even though, in contrast to some of the other prion diseases, it displays additional features of neurodegeneration, such as accumulation of Tau, A-beta and synuclein, demonstrating a complex proteinopathy (44). The fact that, as shown here, the clinical course of this genetic prion disease cannot be manipulated by a CNS inflammatory insult, and that the immune system in these and other genetic patients may not be infected by prions, may have important clinical implications. The most important one, which needs to be tested experimentally, is the high probability that, as opposed to other forms of prion disease, blood from carriers of pathogenic PrP mutations may not be infectious. Interestingly, in a small group of E200K CJD patients who also carry the mutation for FMF (familiar Mediterranean fever), CJD was diagnosed somewhat earlier than in the FMF-negative patients (50–59 years) (45).

The absence of the immune system involvement in genetic and probably some of the sporadic CJD patients may be of great importance in the development of α-prion compounds for patients, and preventive treatment for asymptomatic carriers. Reagents suitable for infectious prion diseases, such as those screened for delaying the accumulation of PrPSc in spleen (47), may not be active in the genetic disease. Indeed, all candidate compounds that have failed over the years in clinical trials

![Figure 5. The effect of EAE on genetic prion disease; summary of current experiments.](image-url)
were tested in *in vitro* or *in vivo* models of infectious prion disease (48,49), while most of CJD patients around the world are of sporadic or genetic etiology. Treatment for these forms of disease need to be developed and tested on models such as our TgMHu2ME199K mice, in which the mechanism of disease propagation may better represent the human disease.

**MATERIALS AND METHODS**

**Induction of EAE to TgMHu2ME199K mice**

The method for induction of MOG-EAE has been described frequently (50,51). In the present experiments, 3-month-old mice from all four types (TgMHu2ME199K/ko, TgMHu2ME199K/wt, wtPrP and koPrP) were immunized with an emulsion containing 300 μg of MOG35-55 (70% purified; synthesized at the Hebrew University, Jerusalem, Israel) solubilized in saline and an equal volume of complete Freund’s adjuvant (CFA; Sigma, Rehovot, Israel) supplemented with 5 mg/ml of heat-killed Mycobacterium tuberculosis H37RA (Difco Laboratories, Detroit, MI, USA). The inoculum (0.2 ml) was injected subcutaneously in both flanks. On the day of inoculation and 48 h later, 100 ng of pertussis toxin (List Biological Laboratories, Campbell, CA, USA) was also administered by intraperitoneal injection.

**EAE scoring system**

Mice were observed daily from Day 6 post induction and throughout the acute phase EAE, for the appearance of neurological symptoms. Mice were scored as follows: 0, asymptomatic; 1, partial loss of tail tonicity; 2, limp tail; 3, impaired righting reflex; 4, hind limb weakness (ataxia); 5, complete hind limb paralysis; 6, moribund or dead (27).

**TgMHu2ME199K scoring system**

Mutant Tg mice from both lines (TgMHu2ME200K/ko and TgMHu2ME200K/wt) were followed up twice a week for the appearance of spontaneous neurological disease. Mice were scored for disease severity and progression according to the scale of clinical signs as previously described (28). Briefly, partial hind limbs weakness = 1, significant hind limb’s weakness or paralysis = 2, full paralysis in one limb = 3, full paralysis in both limbs = 4, death = 5 (28). Mice were sacrificed according to the ethical requirements of the Hebrew University Animal Authorities when too sick or paralyzed to reach food and water, or after losing 20% body weight.

**Brain and spleen fractionation**

Half a milliliter of 10% homogenates in 10 ml Tris–HCl, pH 7.4/0.3 M sucrose from designated animals were subjected to centrifugation at 18,000 rpm for 15 min at 4°C. Subsequently, each supernatant was subjected twice to ultracentrifugation (45,000 rpm for 1 h at 4°C) to eliminate traces of membranes from the soluble protein fraction. The initial pellet containing membrane proteins was resuspended in 2% sarkosyl before ultracentrifugation in 45,000 rpm for 1 h at 4°C. The detergent soluble fraction and the pellet of membrane proteins, together with the initial supernatant, were digested in the presence or absence of 40 μg/ml PK and immunoblotted with aPrP pAb RTC.

**Statistical studies**

Analyses were performed with the Sigma-Stat software package (SPSS, USA). Graphs represent the means of clinical scores of groups of mice. The differences between experimental groups were assessed by one-way analysis of variance, followed by the Mann–Whitney *U* test.

**Pathology**

Four-micrometer-thick sections of formalin fixed, paraffin-embedded brains were evaluated for the presence of mutant PrP aggregation using α-PrP RTC antibody (28,33) and counterstain with hematoxylin. Demyelination was evaluated using Luxol fast blue/periodic acid Schiff (PAS) (52).

**Conflict of Interest statement.** None declared.

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