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Disease-Associated Prion Protein in Neural and Lymphoid Tissues of Mink (*Mustela vison*) Inoculated with Transmissible Mink Encephalopathy

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Summary

Transmissible spongiform encephalopathies (TSEs) are diagnosed by immunodetection of disease-associated prion protein (PrP^d). The distribution of PrP^d within the body varies with the time-course of infection and between species, during interspecies transmission, as well as with prion strain. Mink are susceptible to a form of TSE known as transmissible mink encephalopathy (TME), presumed to arise due to consumption of feed contaminated with a single prion strain of ruminant origin. After extended passage of TME isolates in hamsters, two strains emerge, HY and DY, each of which is associated with unique structural isoforms of PrP^{TME} and of which only the HY strain is associated with accumulation of PrP^{TME} in lymphoid tissues. Information on the structural nature and lymphoid accumulation of PrP^{TME} in mink is limited. In this study, 13 mink were challenged by intracerebral inoculation using late passage TME inoculum after which brain and lymphoid tissues were collected at preclinical and clinical time points. The distribution and molecular nature of PrP^{TME} was investigated by techniques including blotting of paraffin wax-embedded tissue and epitope mapping by western blotting. PrP^{TME} was detected readily in the brain and retropharyngeal lymph node during preclinical infection with delayed progression of accumulation within other lymphoid tissues. For comparison, three mink were inoculated by the oral route and examined during clinical disease. Accumulation of PrP^{TME} in these mink was greater and more widespread, including follicles of rectoanal mucosa-associated lymphoid tissue. Western blot analyses revealed that PrP^{TME} accumulating in the brain of mink is structurally most similar to that accumulating in the brain of hamsters infected with the DY strain. Collectively, the results of extended passage in mink are consistent with the presence of only a single strain of TME, the DY strain, capable of inducing accumulation of PrP^{TME} in the lymphoid tissues of mink but not in hamsters. Thus mink are a relevant animal model for further study of this unique strain, which ultimately may have been introduced through consumption of a TSE of ruminant origin.

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Conflict of Interest The authors declare no financial or personal conflicts of interest with this work.

Keywords

prion; transmissible mink encephalopathy; western blot; immunohistochemistry

Introduction

Transmissible spongiform encephalopathies (TSEs), also known as prion diseases, are slowly progressive fatal neurodegenerative diseases and include variant Creutzfeldt-Jakob disease and kuru in man, scrapie in sheep and goats, chronic wasting disease (CWD) in cervids, several strains of bovine spongiform encephalopathy (BSE) in cattle, and transmissible mink encephalopathy (TME) in mink (Colby and Prusiner, 2011). All forms of TSE are accompanied by hallmark, yet variable, patterns of spongiform change in the central nervous system (CNS) and accumulation of disease-associated forms of prion protein (PrP^d). In general, PrP^d are conformational isoforms of the host cellular prion protein (PrP^c) comprised of a partially protease-resistant core (PrP^{res}) and capable of promoting self-replication (Caughey *et al.*, 2009; Soto, 2011). Different prion disease phenotypes have been recognized in some species and, in part, have given rise to the recognition of prion strains (Collinge and Clarke, 2007; Cobb and Surewicz, 2009; Telling, 2010). In many instances strain discrimination is by recognition of unique profiles of clinical disease, lesion distribution and PrP^d accumulation in the natural host, but may also involve determination of interspecies transmission range. In mink, the possible presence of multiple TME strains was only recognized after serial transmission to hamsters (Kimberlin *et al.*, 1986; Marsh *et al.*, 1991).

In the USA and other countries, TME is a rare disease that occurs as localized epidemics in farm-raised mink (Liberski *et al.*, 2009). The origin of TME is not known, but it has long been suggested to be an exotic disease of mink resulting from ingestion of prion-infected tissue from ruminants (Marsh and Hadlow, 1992). Indeed, scrapie from sheep, BSE from cattle and CWD from elk can be transmitted to mink by experimental inoculation (Hadlow *et al.*, 1987; Marsh *et al.*, 1991; Robinson *et al.*, 1994, 1995; Hamir *et al.*, 2006; Harrington *et al.*, 2008). Modern investigative research now includes extensive use of immunoassays as well as interspecies transmission studies to characterize TSE strains more fully (Wadsworth and Collinge, 2011). In the case of TME, such techniques have been used to probe the neurobiology of the emergence of two strains following serial passage in hamsters (Bessen and Marsh, 1994; Marsh and Bessen, 1994). Similar investigations of TME passaged up to three times in mink, which may also represent a secondary host species, has been limited (Hamir *et al.*, 2006; Harrington *et al.*, 2008).

The aim of the present study was to characterize the time course and distribution of neuropathology and the biochemical nature and tissue distribution of PrP^{TME} accumulation in fourth and fifth passage mink infected experimentally by the intracerebral (IC) and oral routes.

Materials and Methods

Animals

Twenty-six male and female black American mink (*Mustela vison*) of the non-Aleutian disease phenotype (Marsh *et al.*, 1976) with no history of TME were purchased from a commercial breeder in Washington State. Animals were cared for under the guidelines of the Washington State University Institutional Animal Care and Use and Biosafety committees. Animals were housed and managed as previously described (Harrington *et al.*, 2008).

Experimental Design and Animal Procedures

Mink were challenged with mink-passaged inocula of the Stetsonville isolate of TME (Marsh *et al.*, 1991). Brain homogenates made from third- and fourth-passage mink were used as TME inocula, each at a final dilution of 10 % (w/v) in saline. The third-passage TME inoculum was the remaining entirety of a kind gift from Dr. A. Hamir (National Animal Disease Center, US Department of Agriculture, Ames, Iowa, USA) while the fourth-passage TME inoculum was generated in-house from a mink that developed terminal disease 173 days after receiving the third-passage TME inoculum by the IC route. Similar to the original preparation of the third-passage TME inoculum (Hamir *et al.*, 2006), fourth-passage TME inoculum and TME-negative control inoculum (from non-exposed, normal mink) were prepared from half brain samples (including brainstem, cerebellum and cerebrum) that had been stored at -80°C . Half brain samples were prepared individually by fine mincing, trituration in sterile saline and filtering through sterile gauze. The filtered homogenates were brought to a final dilution of 10 % (w/v) in sterile saline and stored at -80°C . After addition of gentamycin (100 $\mu\text{g/ml}$) and a three phase, hot water bath treatment (80°C for 15 min, 37°C for 60 min, 80°C for 15 min), no bacterial contamination was detected by culture on 10 % sheep blood agar.

Mink were challenged by either the IC route using 100 μl of third-passage TME inoculum ($n = 7$ recipients) or fourth-passage TME inoculum ($n = 6$ recipients), or were challenged by the oral route using 600 μl of the fourth-passage TME inoculum ($n = 3$ recipients). IC inoculation was performed under general anaesthesia as described previously (Harrington *et al.*, 2008). Oral inoculation was accomplished by observing ingestion of a small tuna fish meal into which TME inoculum had been pre-mixed. Mink inoculated with third- or fourth-passage TME inoculum represent the respective fourth and fifth experimental passage of the original Stetsonville isolate (Marsh *et al.*, 1991). Non-exposed negative control animals included non-inoculated mink ($n = 5$) and mock IC-inoculated mink ($n = 5$), which received an equivalent original wet weight of TME-negative control inoculum. All animals were monitored weekly for development of clinical signs and necropsy procedures were performed as described by Harrington *et al.* (2008).

Histological and Immunohistochemical Procedures

All histology and immunohistochemistry (IHC) procedures were performed on formalin-fixed, paraffin wax-embedded tissues as described by Harrington *et al.* (2008) with the following minor modifications. Detection of PrP^{TME} was performed using the IgG1 monoclonal antibody (mAb) F99/97.6.1 (O'Rourke *et al.*, 2000) at a final concentration of 5 $\mu\text{g/ml}$ for lymph node and spleen sections, and at 2.5 or 5 $\mu\text{g/ml}$ on brain sections as needed to maximize visualization of brain regions having respectively relatively high or low chromogen reactions. IHC was also performed for glial fibrillary acidic protein (GFAP) using a rabbit polyclonal antibody (CP040C, Biocare Medical; Concord, California, USA) at a final dilution of 1 in 600. The peroxidase substrates, 3-amino-9-ethylcarbazole (AEC) and 3, 3'-diaminobenzidine (DAB) were used to 'visualize' immunolabelled PrP^{TME} and GFAP, respectively. All sections were counterstained using haematoxylin. All haematoxylin and eosin (HE)-stained and immunohistochemically labelled brain sections were evaluated by a pathologist (RDH) without knowledge of the animal's inoculation group or clinical status. These sections were evaluated for severity of vacuolation, PrP^{TME} deposition and astrocytic gliosis.

Positive control tissue sections included the brain and lymph node from TSE-infected mink and sheep. Negative control tissue sections included the brain and lymph node of age-matched non-inoculated and mock IC-inoculated normal mink, as well as from normal sheep. Additional checks for antibody epitope specificity were performed using positive

tissue sections from TME IC- or orally-inoculated mink tissues in either the absence of primary antibody or by primary antibody substitution with an epitope-irrelevant IgG1 mAb or rabbit polyclonal antibody (Ventana Medical Systems; Tucson, Arizona, USA).

Tissue Blotting

Analysis of paraffin wax-embedded tissue by blotting was carried out with modification to procedures originally described for detection of PrP^{res} in fixed brain sections from people, mice, cattle and sheep (Schulz-Schaeffer *et al.*, 2000). Sections (5 µm) of brain or lymphoid tissues from mink were allowed to adhere to 0.45 µm nitrocellulose membranes (GE Healthcare systems, Pittsburgh, Pennsylvania, USA) pre-wetted in distilled water. Membranes were dried at 55°C (minimum of 30 min), dewaxed in xylene (twice for 10 min each) and allowed to dry overnight. Membranes were rehydrated in 1× tris-buffered saline (TBS) to which 0.05% tween 20 was added (TBST). Rehydrated membranes were digested for 1 h at 55°C using 50 µg/ml proteinase K (PK) in TBST, then rinsed in TBST (three times, 5 min each) and denatured for 10 min at 55°C using 8 M urea in 2-mercaptoethanol (8% 2-ME in 125 mM Tris HCl/6 mM EDTA). After rinsing in TBST (three times, 5 min each), membranes were blocked sequentially using 1× solutions of casein in TBST (1 h), avidin and biotin blocking solutions in TBS (10 min each), and dual endogenous enzyme block (DakoCytomation Inc., Carpinteria, California, USA; 10 min) with intervening rinses in diluent (5 min each) and a final rinse in TBST. Immunoassay for the PK-resistant core of PrP^d (i.e. PrP^{res}) was performed by sequential incubation with primary mAb F99/97.6.1 (3.5 µg/ml, 1 h) and a biotinylated secondary anti-mouse antibody (1.5 µg/ml for 30 min), both diluted in 1× casein blocker and followed by rinses in TBST (twice, 5 min each) and a final rinse in 1× casein blocker. Chromogen labelling of bound antibody complex was performed at room temperature using ABC-AmP (30 min; Vector Laboratories, Burlingame, California, USA) and 5-bromo-4-chloro-3-idolyl phosphate/nitroblue tetrazolium substrate (pH 9.5, 5–20 min; BCIP/NBT substrate kit) according to the manufacturer's recommendations. Digital images were acquired under uniform settings and processed using the ImageJ-based open source processing package, Fiji (<http://pacific.mpi-cbg.de/>) (Abramoff *et al.*, 2004).

Western Blotting

Western blotting was performed as previously described (Spraker *et al.*, 2004; Alverson *et al.*, 2006; O'Rourke *et al.*, 2011) with molecular mass estimation using commercial software (AlphaEase FC, Alphaimager; San Leandro, California, USA). Three regions of mink PrP were targeted for epitope mapping using anti-prion protein mAbs for which the epitopes are known (Krasemann *et al.*, 1996; Korth *et al.*, 1997; O'Rourke *et al.*, 2000; Langeveld *et al.*, 2006; Yuan *et al.*, 2008). Fig. 6 summarizes the approximate binding positions and epitope sequence identities with mink PrP. In addition to mAb F99/97.6.1, the following mAbs were used: 12B2 (a kind gift of J. Langeveld, Central Institute for Animal Disease Control, Lelystad, The Netherlands), P4 and L42 (R-BioPharm Inc., Washington, Missouri, USA), 8G8 (Cayman Chemical Company, Ann Arbor, Michigan, USA), 1E4 (Cell Sciences Inc., Canton, Massachusetts, USA) and 6H4 (Prionics USA Inc., La Vista, Nebraska, USA).

Results

Clinical Disease and Survival

A summary of the sampling times, presence of clinical disease and accompanying assessments of neural spongiform change and neural and lymphoid tissue accumulation of PrP^{TME} is given in Table 1. Seven of the 13 TME IC-inoculated mink were killed at preclinical time points or developed intercurrent disease. Clinical signs of TME disease were allowed to develop in the remaining six IC-inoculated mink and three mink challenged by the oral route. Once clinical signs were recognized, all cases of TME progressed over 1–3

weeks. Prominent neurological signs were consistent in animals inoculated by different routes and included lethargy progressing to somnolence, ataxia characterized by a paddling hindlimb gait, and hindlimb weakness progressing to paresis, which rendered the animal unable to return to its nest box (~30 cm above the cage floor) at which point the animals were humanely destroyed. Signs consistent with TME disease did not develop in any negative control mink. Negative control mink were killed electively or due to intercurrent disease at the following time points: non-inoculated mink at 122, 224, 496, 497 and 498 days; mock IC-inoculated mink at 1, 1, 180, 572 and 1548 days post inoculation (dpi).

TME IC-inoculated mink with clinical signs survived for 163–212 dpi whereas mink challenged by the oral route survived 274–498 dpi. In mink with clinical signs challenged by the IC-route, survival times were evenly intermixed between fourth- and fifth-passage mink (passage group from shortest to longest survival: 5th, 4th, 5th, 4th, 5th and 5th). The survival function curve for TME IC-inoculated mink was significantly shorter (Wilcoxon chi-square = 5.19, $P = 0.0227$) than the curve for mink challenged by the oral route. Mean survival times and standard errors were 190 ± 7 dpi for IC-inoculated mink and 377 ± 65 dpi for mink challenged by the oral route.

CNS Histopathology and PrP^{TME} Accumulation

Spongiform change (vacuolar degeneration) was not observed in negative control mink (Fig. 1a) or in TME IC-inoculated mink evaluated at the preclinical time points of 36–73 dpi (Fig. 1d). Spongiform change was observed in two mink with preclinical disease examined at 122 dpi (Figs. 1, b and e) consisting chiefly of a few ~5 mm foci within the cerebrocortical grey matter, within which 10–20 μm round vacuoles occurred at low density within the neuropil and rarely in an intracellular location. Advanced spongiform change was present in mink with terminal clinical disease, including IC-inoculated mink (163–212 dpi; Figs. 1, c and f) and mink challenged by the oral route (274–498 dpi). In mink with terminal TME disease, spongiform change was present throughout the grey matter of the cerebral cortex and consisted of 10–40 μm round vacuoles at high density within the neuropil and in an intracellular location, in some cortical regions coalescing to produce a sponge- or lace-like appearance. Astrocytic gliosis was also evident in TME IC-inoculated mink as compared with mock IC-inoculated control mink (Supplemental Fig. 1).

As detected in the brain by IHC, the progression of PrP^{TME} accumulation was similar to that for spongiform change. In TME IC-inoculated mink, PrP^{TME} was not observed in mink with preclinical disease evaluated at 36–73 dpi (Fig. 1d), but was observed in both mink with preclinical disease examined at 122 dpi (Fig. 1e). At 122 dpi, PrP^{TME} was mainly distributed as small dispersed foci within glial cells and the neuropil of the cerebral cortex, whereas PrP^{TME} was dispersed more regionally and increased in density during terminal disease at 163–212 dpi (Fig. 1f). The relative abundance of prp^{TME} was greatest within several regions of the cerebral cortical grey matter, less within the hippocampus, thalamus and pons, even less within the caudal regions of the medulla oblongata, including the obex, and only as rare pinpoint foci within the cerebellum. PrP^{TME} was not detected in either negative control group of non-exposed mink.

PrP^{TME} Accumulation in Lymphoid Tissues

In TME IC-inoculated mink, accumulation of PrP^{TME} was not detected within the lymphoreticular system until 122 dpi. At this time point of preclinical disease, PrP^{TME} accumulation was detected within the follicles of the retropharyngeal lymph node (Fig. 2a), but was not detected within other lymphoid tissues (Table 1; e.g. mesenteric lymph node, Fig. 2c). At the time of terminal clinical disease (163–212 dpi), detection in lymphoid tissues was variable. Immunolabelling consistently included the retropharyngeal (Fig. 2b)

and mesenteric (Fig. 2d) lymph nodes, but was variable with minimal detection in the gut-associated lymphoid tissue (Fig. 3a) and spleen (Fig. 3b). In mink evaluated at terminal time points, the distribution and accumulation of PrP^{TME} within the lymphoreticular system was similar between IC-inoculated and orally-challenged mink, except for the rectoanal mucosa-associated lymphoid tissue (RAMALT, Table 1) where PrP^{TME} was only detected in mink challenged by the oral route (Fig. 3c).

Tissue Blot Detection of PrP^{res} in Brain and Lymphoid Tissues

Thin sections of brain, retropharyngeal and mesenteric lymph nodes of three TME IC-inoculated mink with clinical disease (173–212 dpi) were prepared for tissue blotting and IHC analysis. Each tissue blot reaction was run simultaneously with a similar tissue section from a non-inoculated mink (224 dpi). PrP^{TME} was detected by IHC in the brain (Fig. 4a), retropharyngeal (Fig. 4d) and mesenteric (not shown) lymph nodes of mink with clinical signs. PrP^{res} was readily detected by tissue blot analysis in the corresponding tissue sections from the mink with clinical signs (brain, Fig. 4b; retropharyngeal lymph node, Fig. 4e), but was not detected in the brain and lymph node sections from a non-inoculated mink (brain, Fig. 4c; retropharyngeal lymph node, Fig. 4f).

Western Blot Analyses of PrP^{res}

As detected by western blotting using mAb 99/97.6.1, three major bands of PrP^{res} were detected in the brain of sheep with classical scrapie (Fig. 5a, lane 1; 5b, lanes 1 and 3) and in the brain (Fig. 5a, lane 2; 5b, lanes 2 and 4) and retropharyngeal lymph node (Fig. 5a, lane 3) of TME IC-inoculated mink, but were not detected in the brain of a mock IC-inoculated mink (Fig. 5b, lane 5) or the retropharyngeal lymph node of a non-inoculated mink (Fig. 5a, lane 4). The molecular masses of PrP^{res} bands derived from the brain of TME IC-inoculated mink (hereafter, TME-PrP^{res}) were 25.7–26.2, 21.4–22.0 and 17.5–17.9 kDa, consistently ~1 kDa less than PrP^{res} bands derived from the brain of sheep with classical scrapie (hereafter, Sc-PrP^{res}; 27.0–27.7, 22.4–22.7, 18.8–19.0 kDa). The molecular masses of non-digested total PrP and TME-PrP^{res} bands from mink were consistent and not dependent on route of inoculation (Fig. 5c). At these equivalent loading concentrations, an additional low molecular mass non-digested PrP band was noted only in mink with TME, whether induced by IC or oral inoculation, and equivalent in mass to the lowest TME-PrP^{res} band.

Epitope mapping of brain-derived TME-PrP^{res} from mink was conducted using mAbs with known epitopes and corresponding to one of three regions of the mink PrP sequence (Fig. 6a). The results were compared with those for brain-derived Sc-PrP^{res} from a sheep with classical scrapie. Within the most N-terminal region (region 1; Fig. 6b), labelling was observed when using mAbs 12B2, P4, 8G8 and 1E4 to detect undigested PrP and Sc-PrP^{res} bands of sheep. In contrast, region 1 mAbs did not label TME-PrP^{res} from mink; however, only mAb 12B2 labelled undigested mink PrP. Labelling of undigested PrP and PrP^{res} was observed for mAbs binding epitopes in the middle (region 2; mAbs 6H4 and L42) and C-terminal (region 3; mAb F99/97.6.1) regions of PrP, whether derived from the brain of sheep with classical scrapie or mink with TME (Fig. 6c).

As shown in Fig. 7, the putative PK cleavage sites (vertical lines) and previously described N-terminal amino acids (triangles) for PrP^{res} peptides were mapped along the mature PrP sequences (orange arrows) of sheep, mink and hamster. As measured in the current study for sheep with classical scrapie, the molecular mass and preservation of epitope regions 1–3 (solid green bars) are consistent with Sc-PrP^{res} peptides (sheep, yellow arrows) having N-terminal amino acids at either of two (solid triangles) or four previously described positions (Gielbert *et al.*, 2009). In mink with TME, two TME-PrP^{res} peptides (mink, yellow arrows) having N-terminal amino acids located at putative PK cleavage sites are consistent with the

observed selective loss of labelling with a region 1 epitope (12B2, open green bar) and the ~1 kDa difference in molecular mass relative to Sc-PrP^{res}. These characteristics of TME-PrP^{res} are most consistent with the major PrP^{res} peptides that have been associated with the DY strain (hamster, lower yellow arrows), rather than the HY strain (hamster, upper yellow arrows), of TME passed in hamsters (Bessen and Marsh, 1994).

Discussion

In the characterization of TSE diseases, comparisons of clinical signs and course of disease, neuropathology and characterizations of PrP^d after transmissions within and between species, have aided in the recognition of prion strains. In this regard, the continued study of TME has contributed not only to the recognition of multiple TME strains, but also to mechanisms underpinning disease phenotypes. When the original Stetsonville isolate of TME was initially transmitted from mink to Syrian golden hamsters by IC inoculation of brain homogenate (Marsh *et al.*, 1991), only one disease phenotype was observed – typified by hyperexcitability (HY), cerebellar ataxia and a short disease course. A second syndrome, typified by drowsiness (DY) and prolonged disease course, only became evident in a few hamsters upon third passage. Each syndrome has since been characterized (Bessen and Marsh, 1992a, b; Bartz *et al.*, 2000, 2007; Shikiya *et al.*, 2010) in part by differences in the accumulation of unique PrP^{TME} isoforms (PrP^{HY-TME}, PrP^{DY-TME}) in the brain and peripheral lymphoid tissues of hamsters. A similar account of the emergence of two disease syndromes on serial passage of TME in Chinese hamsters has also been described (Kimberlin *et al.*, 1986).

It is plausible that both prion strains are simultaneously present in the brain of mink with TME. However, TME has long been suspected to be an exotic disease of mink and recent research suggests it is caused by transmission of a single strain of BSE (L-type) in cattle (Baron *et al.*, 2007). Interestingly, after limited cloning (third passage) of the Stetsonville isolate of TME in mink (Bartz *et al.*, 2000), two strains still emerge in hamsters. To our knowledge, further serial passage of the Stetsonville isolate in mink has not been reported. In the present study only one clinical syndrome was observed in fourth- and fifth-passage mink, and this was consistent with previous original and third-passage descriptions of clinical signs, disease course, neuropathology and brain accumulation of PrP^{TME} (Bartz *et al.*, 2000; Hamir *et al.*, 2006). Thus, if two strains of TME persist in mink after serial passage, then one remains 'silent' even after extended cloning.

Similar to a previous study (Hamir *et al.*, 2006), limited proteolysis of PrP^{TME} derived from the brain of mink results in a greater reduction in molecular mass than that observed for PrP^{Sc} derived from the brain of sheep with classical scrapie (Thuring *et al.*, 2004; Vidal *et al.*, 2007; Gielbert *et al.*, 2009). The reduction was even greater for PrP^{TME} derived from lymphoid tissues of mink. This relative increased susceptibility to proteolysis is not likely due to C-terminal cleavage since labelling with mAb F99/97.6.1 (epitope region 3) was retained, a finding consistent with the general resistance of PrP^d to C-terminal cleavage (Stahl *et al.*, 1990; Narwa and Harris, 1999). In contrast, loss of labelling with mAb 12B2 could be from direct destruction of this region 1 epitope or from epitope masking induced by a more proximal N-terminal cleavage site (Polymenidou *et al.*, 2008; Zou *et al.*, 2010). Given the previously described N-terminal amino acid profiles of PrP^{Sc} (Gielbert *et al.*, 2009) and the relative differences in PrP^{res} molecular mass and region 1 epitope mapping in the current study, we conclude that brain-derived PrP^{TME} is more susceptible to N-terminal proteolysis, which includes cleavage of the mAb 12B2 epitope. Epitope sequence differences between mink PrP and other region 1 epitopes prevented further discernment of the effects of proteolysis. Nonetheless, the molecular characteristics of PrP^{TME} derived from mink are most similar to those associated with the emergent DY-strain in hamsters. In

contrast to the PrP^{TME} associated with the HY-strain in hamsters, PrP^{TME} associated with the DY-strain is more susceptible to proteolysis, differentially losing ~1–2 kDa more in molecular mass (Bessen and Marsh, 1994) and epitope labelling in the analogous region 1 of PrP (Bessen and Marsh, 1992a).

For many prion diseases, development of immunoassays capable of detecting PrP^d in lymphoid tissues has significantly improved live animal diagnostic and surveillance efforts for several prion diseases, including classical scrapie disease in sheep and goats (O'Rourke *et al.*, 2000; O'Rourke *et al.*, 2002; Ersdal *et al.*, 2003; Valdez *et al.*, 2003; Caplazi *et al.*, 2004) and CWD in cervids, especially in mule and white-tailed deer (Spraker *et al.*, 2002, 2009; Keane *et al.*, 2008; Keane *et al.*, 2009). However, detection of significant peripheral accumulation is variable or does not typically occur in other TSEs, including BSE in cattle (Fraser and Foster, 1994; Wells and Wilesmith, 1995; Wells *et al.*, 1998), atypical or Nor98 scrapie in sheep (Benestad *et al.*, 2003) and some forms of human TSE (Ironside *et al.*, 2002).

This creates a diagnostic quandary while also generating interest in comparative lymphoid involvement between TSE strains and disease hosts. It is interesting that although BSE in cattle is not normally associated with lymphoid accumulation of PrP^{BSE}, lymphoid accumulation is observed if cattle are inoculated experimentally with BSE by the oral route (Terry *et al.*, 2003; Iwata *et al.*, 2006). Moreover, lymphoid accumulation of PrP^d in a variant form of human TSE is thought to result from transmission of BSE from cattle to man (Bruce *et al.*, 1997; Scott *et al.*, 1999), originally through the consumption of contaminated foods. Similarly, PrP^{BSE} accumulates in the lymphoid tissues of sheep infected experimentally with BSE (van Keulen *et al.*, 2008). The accumulation of PrP^d in peripheral tissues of mink would therefore not be inconsistent with the effect of transmission of BSE to other species.

Given the potential relevance to interspecies transmission of ruminant TSEs, we further examined the effects of exposure route and time course on PrP^{TME} accumulation in mink. Accumulation of PrP^{TME} was detected readily in several lymphoid tissues of mink following IC inoculation and more so following oral inoculation. To verify that the IHC signal in mink tissues was PrP^{TME}, we successfully adapted the highly-sensitive tissue blot technique (Schulz-Schaeffer *et al.*, 2000) and demonstrated co-localization of PrP^{res} with PrP^{TME} in the brain and lymphoid tissues of mink. In IC-inoculated mink, the earliest accumulation of PrP^{TME} in the brain was accompanied by accumulation within the retropharyngeal lymph node, suggesting the original inoculum was not completely contained within the CNS, but escaped early into the local lymphatic system. This conclusion is supported by the delayed accumulation seen in more distant lymphoid tissues of mink in the present study, and is similar to a delayed lymphoid tissue distribution observed in hamsters when the HY strain of TME was injected directly into the tongue (Bartz *et al.*, 2003). In contrast, lymphoid accumulation of PrP^{TME} does not occur when hamsters are inoculated by any route with the DY strain (Bartz *et al.*, 2005). Thus mink, although presenting a more challenging husbandry problem, are a relevant animal model to the study of this TSE strain.

The results of the present study are most consistent with the presence of a single prion strain after extended passage of the Stetsonville isolate of TME in mink. The PrP^{TME} that accumulates in the brain of mink appears to be most similar structurally to the isoform that accumulates in the brain of hamsters infected with the DY strain. Unlike the DY strain in hamsters, however, the strain passaged in mink is associated with significant accumulation of PrP^{TME} in lymphoid tissues, similar to BSE as passaged in sheep and as the probable cause of a variant TSE in man.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Progressive spongiform change and PrP^{TME} accumulation (red) in the cerebral cortex of mink inoculated by the IC route. (a) The normal cerebral cortex of a non-inoculated negative control mink (122 dpi). (d) Normal cerebral cortex and lack of PrP^{TME} accumulation in a TME IC-inoculated mink at the preclinical time point of 73 dpi. Early spongiform change (b) and PrP^{TME} accumulation (e) in a TME IC-inoculated mink at the preclinical time point of 122 dpi. Advanced spongiform change (c) and PrP^{TME} accumulation (f) in a TME IC-inoculated mink at the clinical time point of 173 dpi. Top row, HE. Bottom row, IHC. Bars, 100 μ m.

Fig. 2. PrP^{TME} accumulation (red) in a retropharyngeal lymph node (a), but not a mesenteric lymph node (c) from a TME IC-inoculated mink at the preclinical time point of 122 dpi. PrP^{TME} accumulation in retropharyngeal (b) and mesenteric (d) lymph nodes from a TME IC-inoculated mink at the clinical time point of 198 dpi. IHC. Bars, 100 μm.

Fig. 3. (a) PrP^{TME} accumulation (red) in gut-associated lymphoid tissue from a TME IC-inoculated mink at the clinical time point of 212 dpi. (b) PrP^{TME} accumulation in lymphoid follicles of the spleen from a TME IC-inoculated mink at the clinical time point of 198 dpi. (c) PrP^{TME} accumulation in rectoanal mucosa-associated lymphoid tissue from a TME orally-inoculated mink at the clinical time point of 274 dpi. IHC. Bars, 100 μm.

Fig. 4.

Co-distribution of PrP^{TME} and protease-resistant TME-PrP^{res} accumulations in the brain and retropharyngeal lymph node. Note the similar distribution of PrP^{TME} (red) and TME-PrP^{res} (pseudocoloured) accumulations in the brain (a versus b) and in a retropharyngeal lymph node follicle (d versus e) from a TME IC-inoculated mink at the clinical time point of 212 dpi. TME-PrP^{res} labelling is not evident in the brain (c) or a retropharyngeal lymph node follicle (f) from a non-inoculated mink (224 dpi). IHC (a, d). Tissue blot (b, c, e and f). Curved lines indicate follicle (d–f) or tissue (c–f) margins. Bars: (a), 2 mm; (d), 100 μm. Pseudocolour bars: (b, c) and (e, f).

Fig. 5.

(a, b) Shown are the PK-resistant PrP bands derived from the brain of a sheep with naturally acquired classical scrapie disease (a, lane 1; b, lanes 1 and 3), from the brains of TME IC-inoculated mink at 173 dpi (b, lane 2) and at 198 dpi (a, lane 2; b, lane 4), and from the retropharyngeal lymph node of a TME IC-inoculated mink at 212 dpi (a, lane 3). PK-resistant PrP bands are not detected in the brain of a mock IC-inoculated mink at 180 dpi (b, lane 5) or in the retropharyngeal lymph node of a non-inoculated control mink at 224 dpi (a, lane 4). (c) Shown are the consistencies in apparent molecular mass of total undigested PrP bands (lanes 1–10) and of PK-resistant PrP bands (lanes 11–16) derived from the brain of mink with TME, whether inoculated by the IC or oral routes. Note also the presence of an additional low molecular mass band in undigested PrP from mink with TME (lanes 1, 3, 5, 7–10) as compared with a mock IC-inoculated mink (lane 2) or non-inoculated mink (lanes 4 and 6). PK-resistant PrP bands are not detected in the brain of non-inoculated mink at 224 and 122 dpi, respectively (c, lanes 17 and 18). In each image, positions of molecular mass markers (kDa) are located along the left margin and lanes are enumerated along the bottom margin. PK, proteinase K; sh, sheep; m, mink; TSE, transmissible spongiform encephalopathy (classical scrapie in sheep, TME in mink); b, brain; l, retropharyngeal lymph node; na, natural exposure; 0, non-inoculated; ic, intracerebral inoculation; po, oral inoculation.

Fig. 6.

(a) Cartoon map of mink PrP (orange bar) indicating putative PK cleavage sites (vertical lines) and regional epitopes (green bars). (b and c) Regional sequence alignments of mink PrP and mAb epitopes are shown along with the results of corresponding western blot analyses. Sequence identities (grey highlighted) and putative PK cleavage sites (triangles) are indicated. In western blot analyses of PrP derived from the brain of a sheep with classical scrapie, note that labelling was retained for mAbs from all three epitope regions after partial digestion of PrP using PK. In contrast, labelling was lost after partial PK digestion of mink PrP for the region 1 mAb 12B2, but not for regions 2 and 3 mAbs; sequence differences appear to prevent any labelling of mink PrP using region 1 mAbs P4, 8G8 and 1E4. In each image, positions of molecular mass markers (kDa) are located along the left margin and lanes are enumerated along the bottom margin. PK, proteinase K; sh, sheep; m, mink; TSE, transmissible spongiform encephalopathy (classical scrapie in sheep, TME in mink); na, natural exposure; 0, non-inoculated; ic, intracerebral inoculation.

Fig. 7.

Shown are cartoons of mature PrP (orange arrows) of sheep, mink and hamsters with locations indicated for putative PK-cleavage sites (vertical lines) and previously reported N-terminal amino acid profiles (triangles). In sheep with classical scrapie, all epitope labelling was retained (filled green bars) after partial PrP digestion using PK, indicating the PK-resistant core of Sc-PrP^{res} (yellow arrows) has an N-terminal amino acid at position 85 or 89 (solid triangles), but not at positions 94 or 96 (open triangles). For mink inoculated experimentally with TME, loss of mAb 12B2 labelling (open green bar) is consistent with TME-PrP^{res} (yellow arrows) having an N-terminal amino acid at or near either of two putative PK-cleavage sites, 94 and 104. The characteristics of PrP^{res} derived from the brain of mink with TME is thus most consistent with the N-terminal amino acid profiles of the brain-derived PrP^{res} associated with the DY-strain (lower yellow arrows), but not the HY-strain (upper yellow arrows), of TME passaged in hamsters.

Table 1

Progression of disease, neural spongiform change and PrP^{TME} accumulation

DPI	Disease status	(HE) Vacuoles	(IHC) Prp ^{TIME}					
			CNS	RPLN	Spleen	MLN	GALT	RAMALT
Intracerebral inoculation								
36, 55, 58, 72, 73	Non-clinical [*]	-	-	-	-	-	-	NA
122, 122	Non-clinical	+	+	+	-	-	-	-
163	Clinical	+++	+++	+++	-	+++	NA	-
173	Clinical	+++	+++	+++	+	+++	NA	-
197, 198, 198	Clinical	+++	+++	+++	+	+++	+	-
212	Clinical	+++	+++	+++	+	+++	++	-
Oral inoculation								
274, 359, 498	Clinical	+++	+++	+++	+	+++	++	++

DPI, days post inoculation; CNS, central nervous system; RPLN, retropharyngeal lymph node; MLN, mesenteric lymph node; GALT, gastrointestinal-associated lymphoid tissue; RAMALT, recto-anal lymphoid tissue; HE, haematoxylin and eosin stain; IHC, immunohistochemistry for PrP^{TME} using mAb F99/9.6.1, AEC substrate and haematoxylin counterstain.

Histological scores: -, within normal limits; +, slight; ++, moderate; +++, marked; NA, sample not available.

*

Intercurrent pneumonia in one mink; intercurrent rectal prolapse in one mink.