Identification of two biologically distinct strains of transmissible mink encephalopathy in hamsters

Richard A. Bessen and Richard F. Marsh*

Department of Veterinary Science, University of Wisconsin-Madison, 1655 Linden Drive, Madison, Wisconsin 53706, U.S.A.

Experimental transmission of the Stetsonville, Wisconsin, U.S.A. source of transmissible mink encephalopathy (TME) to outbred Syrian golden hamsters resulted in two distinct syndromes, termed hyper (HY) and drowsy (DY), that diverge by the third hamster passage. The syndromes differed with respect to clinical signs, incubation period, brain titre, brain lesion profile and pathogenicity in mink. HY hamster TME had an incubation period of 65 ± 1 days and was characterized by clinical signs of hyperaesthesia and cerebellar ataxia. Lethargy and the absence of hyperexcitability or cerebellar ataxia were representative of DY hamster TME which had an incubation period of 168 ± 2 days. At endstage, HY and DY infected animals had brain titres of $10^{9.5}$ LD$_{50}$/g and $10^{7.4}$ LD$_{50}$/g of tissue, respectively, indicating that the replication kinetics of these two strains is different. Hamster TME passaged back into mink revealed that only DY retained mink pathogenicity. This suggests that the DY agent is the major mink pathogen in the Stetsonville TME source that is also pathogenic in hamsters after a long incubation period. The HY agent is likely to be a minor component of the original TME mink brain that replicates more rapidly than DY agent in hamsters, but alone is non-pathogenic in mink. The presence of the HY and DY strains of agent that retain their biological characteristics on repeated hamster passage in the Stetsonville TME source requires that the informational molecule encoding these transmissible agents has the capacity to account for this biological diversity.

Introduction

Transmissible mink encephalopathy (TME) is a rare neurodegenerative disease of ranch-raised mink related to other transmissible encephalopathies in animals and humans. The disease is characterized by long incubation periods followed by progressive neurological dysfunction and death. TME was first described by Hartsough & Burger in 1965 and epidemiological studies indicated that the disease was a foodborne infection caused by an unidentified feed ingredient (Hartsough & Burger, 1965; Burger & Hartsough, 1965). Experimental attempts to transmit sheep scrapie to mink from sources in both the United States (Hanson et al., 1971) and the United Kingdom have been inconsistent with the epidemiology of natural TME (Marsh & Hanson, 1979). In 1985, an outbreak of TME in Stetsonville, Wisconsin, U.S.A. was reported in which fallen dairy cattle were the major animal protein in mink feed (Marsh & Hartsough, 1985; Marsh & Hartsough, 1988; Marsh et al., 1991). Stetsonville TME was inoculated into two Holstein steers, each of which developed a fatal spongiform encephalopathy 18 to 19 months post-infection (Marsh et al., 1991). Upon passage back into mink, bovine brains remained highly pathogenic for mink indicating an absence of a species barrier effect between mink and cattle, and suggesting that an unrecognized scrapie-like disease of cattle may exist in the United States (Marsh & Hartsough, 1985; Marsh et al., 1991).

TME has been transmitted to many animal species including hamsters (Marsh et al., 1969; Kimberlin et al., 1986), subhuman primates (Marsh et al., 1969; Eckroade et al., 1970), skunks, ferrets and raccoons (Eckroade et al., 1973), and sheep and goats (Hadlow et al., 1986). The Hayward source of TME was transmitted to both Syrian golden hamsters (Marsh et al., 1969; Marsh & Kimberlin, 1975) and Chinese hamsters (Kimberlin et al., 1986). In Syrian golden hamsters, initial passage of Hayward TME led to clinical disease characterized by hyperaesthesia, incoordination and cerebellar ataxia (Marsh et al., 1969) but at later passages was characterized by lethargy, slowed voluntary movements and slight incoordination (Marsh & Kimberlin, 1975). Additional animal passage studies with the Hayward TME source concluded that it consists of a mixture of agent subpopulations including a hamster pathogen and a mink–monkey pathogen (Marsh
of TME resulted in the identification of two hamster vacuolization patterns (Kimberlin et al., 1986). Strain variation was believed to be a result of mutation or selection of TME agents at some point during their passage history (Kimberlin et al., 1986).

‘Strains’ have been best studied in mouse scrapie where over 15 have been identified based on the length of incubation periods in mice of defined genotype and on the lesion profile of the brain pathology (Fraser, 1976; Dickinson & Fraser, 1977). Two hypotheses on the nature of these transmissible agents provide an explanation for strain variation. The virino hypothesis states that an agent-specific genomic nucleic acid utilizes host protein as a protective coat to form the infectious agent. Alternatively, the prion hypothesis states that the agent is composed of a host-encoded prion protein that becomes modified during infection; it is likely that prions are devoid of nucleic acid (Prusiner, 1982; Bellinger-Kawahara et al., 1988).

Strain variation in this instance may arise from either differences in the primary amino acid sequence of the prion protein (Westaway et al., 1987; Carlson et al., 1989) or different post-translational modifications (Prusiner et al., 1990).

This report describes the transmission of the Stetsonville source of TME to Syrian golden hamsters and the identification of two hamster TME strains that have different biological properties based on length of incubation period, clinical disease, endpoint brain titres, brain lesion profile and mink pathogenicity.

**Methods**

*Source of TME and animal inoculations.* Mink brain was obtained from a 1985 incident of TME in Stetsonville, Wisconsin, U.S.A. as described previously (Marsh et al., 1991). Neonatal mink were obtained from the University of Wisconsin Mink Unit and weanling, outbred male Syrian golden hamsters (LVK/LAK) purchased from Harlan Sprague Dawley. Individual brains stored at -80 °C were homogenized in TBS (20 mm-Tris–HCl pH 7.4, 100 mm-NaCl) containing 0.32 M-sucrose using disposable, sterile vials and syringes to avoid contamination of inocula. Animals were intracerebrally inoculated into the right cerebral hemisphere with 0.05 ml of 1%, 5% or 10% (w/v) brain homogenates from either a TME-infected mink or from individual hamsters. No pools of brain tissue were used for inoculation in these experiments. Hamsters and mink were observed twice weekly for clinical disease. Incubation periods were measured as the length of time between inoculation and the onset of progressive clinical disease. Endpoint brain titres were calculated according to the method of Kärber (Parker, 1959). Animals were handled and cared for according to NIH and University of Wisconsin Research Animal Resources guidelines.

*Histology.* Brain tissue was fixed in freshly prepared 10% formalin, imbedded in paraffin, sectioned and stained with haematoxylin and cosin.

**Results**

*Transmission of TME to Syrian golden hamsters.*

Outbred, weanling male Syrian golden hamsters were intracerebrally inoculated with a 5% brain homogenate from a mink affected with TME (Marsh et al., 1991). On first passage (Fig. 1), the incubation period was 15 to 16 months and was shortened to 209 ± 4 days on the second hamster passage. Only eight of 14 animals were affected on second passage and the remainder were sacrificed at 230 days post-infection. Onset of clinical disease was characterized by hyperaesthesia, tremors of the head and body, incoordination and cerebellar ataxia. As the disease progressed, animals became somnolent, could no longer remain upright and became severely debilitated several days before death. The clinical course averaged 3 weeks in duration.
Initially, third passage hamster TME had an incubation period of 138 ± 5 days in seven of 12 animals and the survivors were sacrificed at 170 days post-infection (data not shown). When the third hamster passage was repeated with the same two individual brains and hamsters were observed for a longer period of time, two clinical syndromes were observed for each brain inoculum. One showed predominant clinical signs of hyperaesthesia and ataxia, as seen previously, and the second was characterized by lethargy after longer incubation periods (Fig. 1, 8HD and 664D). Animals in the latter group became increasingly drowsy, voluntary movements were slowed and they lacked coordination when aroused but did not become hyperexcitable or exhibit cerebellar ataxia. A waddling gait, kyphosis, and ptosis of the eyelids were often observed. These two syndromes were termed hyper (HY) and drowsy (DY) to designate their major clinical signs.

Upon fourth hamster passage, all HY inocula produced uniform syndromes after an incubation period of 72 ± 1 days. Passage of two individual DY brains resulted in 15 of 15 hamsters inoculated with one (Fig. 1, 664D) of the brains manifesting the DY syndrome after 173 ± 3 days, and animals inoculated with the other brain (Fig. 1, 8HD) developing the HY syndrome after incubation periods of 109 ± 2 days. Transmission did not occur in three of 42 hamsters due to intercurrent deaths prior to the earliest clinical signs of disease. This was the only point at which reversion of DY to HY TME was observed. In all other cases the HY and DY agents were separately passaged in hamsters and remained biologically distinct from one another. By the fifth hamster passage, HY and DY TME produced stable incubation periods of 65 ± 1 days and 168 ± 2 days, respectively (Fig. 1).

**Infectivity titres**

Serial 10-fold dilutions of HY and DY brain from fifth passage clinically affected animals were injected intracerebrally into weanling hamsters and endpoint titres measured 400 days post-infection (Parker, 1959). HY and DY brains had titres of $10^{9.5}$ LD$_{50}$/g and $10^{7.4}$ LD$_{50}$/g of tissue, respectively.

**Examination of agent competition or interference**

The sudden appearance of DY hamster TME at third passage could be due to competition of the HY and DY agents. Competition between short and long incubation period mouse scrapie agents has been reported in inbred strains of mice (Dickinson et al., 1972, 1975). For this to occur, the DY agent would have to be present in the brain of second hamster passage animals that clinically displayed the HY syndrome. Accumulation of the DY agent in the initial passages at levels too low to produce disease could compete with the HY agent upon subsequent passages and block the HY syndrome. To test this hypothesis, hamsters were intraperitoneally inoculated with HY hamster hamster brain from either HY, DY or a mixture of HY and DY (Table 1). Animals co-infected with both strains had similar incubation periods to those animals infected only with HY hamster TME. Under the testing conditions employed, no interference or blocking of the HY agent occurred in animals co-infected with both the DY and HY agents. Therefore, the appearance of DY TME at third hamster passage is probably not due to competition between different strains of the agent.

**Mink pathogenicity of HY and DY hamster TME**

HY and DY hamster TME were intracerebrally inoculated into neonatal mink to investigate the effect of strain selection on mink susceptibility (Table 2). The original Stetsonville mink brain had an incubation period of 127 ± 1 days compared to 139 ± 1 days with first hamster passage brain (Table 2, 2HD). DY hamster TME retained its mink pathogenicity through at least four passages with incubation periods of 158 ± 1 days (664D) and 160 ± 1 days (247D). In contrast, fourth, fifth and

**Table 1. Assay for competition between HY and DY hamster TME agents**

<table>
<thead>
<tr>
<th>Inoculum*</th>
<th>Incubation period (days ± S.E.M.)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>HY</td>
<td>106 ± 3 (n = 29)</td>
</tr>
<tr>
<td>HY:DY (1:1 mixture)</td>
<td>115 ± 5 (n = 13)</td>
</tr>
<tr>
<td>DY</td>
<td>&gt;320 (n = 21)</td>
</tr>
</tbody>
</table>

* Inoculated intraperitoneally with 0.1 ml of a 1% (w/v) third passage hamster brain homogenate.
† n, Number of animals inoculated.

**Table 2. Pathogenicity of hamster-passaged HY and DY TME in mink compared to the Stetsonville mink brain inoculum**

<table>
<thead>
<tr>
<th>Inoculum*</th>
<th>Hamster passage</th>
<th>Incubation period (days ± S.E.M.)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>St TME‡</td>
<td>0</td>
<td>127 ± 1 (n = 8)</td>
</tr>
<tr>
<td>2HD‡</td>
<td>1</td>
<td>139 ± 1 (n = 6)</td>
</tr>
<tr>
<td>8HD</td>
<td>3</td>
<td>166 ± 4 (n = 7)</td>
</tr>
<tr>
<td>664D</td>
<td>3</td>
<td>158 ± 1 (n = 6)</td>
</tr>
<tr>
<td>64H</td>
<td>4</td>
<td>&gt;440 (n = 5)</td>
</tr>
<tr>
<td>247D</td>
<td>4</td>
<td>160 ± 1 (n = 9)</td>
</tr>
<tr>
<td>279H</td>
<td>5</td>
<td>&gt;300 (n = 5)</td>
</tr>
<tr>
<td>869H</td>
<td>7</td>
<td>&gt;820 (n = 7)</td>
</tr>
</tbody>
</table>

* Neonatal mink inoculated intracerebrally with 10% brain homogenates from individual animals.
† n, Number of animals inoculated.
‡ St, Stetsonville; H, hyper; D, drowsy.
more intense with microvacuolation most prominent in the neocortex. One remarkable finding was a focal area of severe spongiform degeneration adjacent to the pyramidal layer of the hippocampus, near the lateral ventricles, which was always present in hamsters affected with the DY syndrome (Fig. 2) and never with HY.

As the number of serial passages of the HY syndrome increased, the lesion intensity decreased. Perhaps because of fewer passages, this same observation has not yet been made for the DY syndrome where the length of clinical illness seems to be an important factor in determining the severity of spongiform degeneration. Those animals killed in the early stage of clinical disease had only minimal lesions. No attempt was made in this study to assess the onset or distribution of the reactive astrocytic response that has been previously shown to precede spongiform degeneration in TME- and scrapie-affected hamsters (Marsh & Kimberlin, 1975).

Discussion

We report that the Stetsonville TME source contains at least two distinct strains of agent. The DY strain appears to be the major mink pathogen that produced a long incubation TME-like disease in hamsters, whereas the HY strain is a minor component of the original source but predominates in the hamster model. The HY strain produced a short incubation period in hamsters characterized by hyperexcitability and ataxia. In this respect, HY resembles scapie strain 263K, another short incubation hamster pathogen (Kimberlin & Walker, 1977). Since hamster scrapie is studied in the same facilities in which the TME transmissions were performed, it is important to ensure that the HY syndrome does not represent inadvertent contamination with scrapie. The following observations suggest that this has not occurred. (i) Contamination with scrapie at first or second passage would have resulted in subsequent incubation periods of 2 months on passage, not 7 and 4 months, (ii) the neuropathology observed on early passage of the HY syndrome is much more severe than hamster scrapie, where the disease produces only minimal lesions on high passage (unpublished results), (iii) early passages of the HY syndrome were pathogenic for mink (Table 2, 2HD and 8HD), hamster scrapie is non-pathogenic and (iv) we have not observed inadvertent transmission of hamster scrapie to uninoculated animals in our colony nor to hamsters receiving inocula with low infectivity requiring observation for greater than 18 months.

The transmission of two distinct clinical syndromes to hamsters after decreasing incubation periods at each
serial passage until stabilizing after the fourth passage is consistent with host selection of agent strains from a mixture of subpopulations (Kimberlin & Walker, 1978a). We do not believe that host-induced modification or mutation of strains can explain the occurrence of new biological variants (Bruce & Dickinson, 1979) since DY TME retains mink pathogenicity whereas HY TME loses its ability to cause disease in mink. Additionally, we observed a reversion of some third passage DY brain inocula on the next serial passage indicating that if a mutation did occur it was very unstable. The revertants can be more easily explained by the presence of HY TME in the brains of the animals exhibiting the DY syndrome and, upon subsequent passages, disease being caused by the shorter incubation HY pathogen. Therefore, we believe that the original Stetsonville source of TME contained a mixture of HY and DY agents that are separated by host selection and amplified by serial passaging, and that these strains do not arise due to mutation or host modification.

The absence of the DY syndrome until third hamster passage may have been due to premature termination of the experiments. Hamsters that were not clinically affected (manifested as the HY syndrome) during the initial early passages were sacrificed but may have expressed the longer incubation period DY disease if permitted to survive. It was only when the surviving unaffected hamsters from the third passage were observed for a longer period of time that the DY syndrome was observed. Although first passage hamster TME produced only the HY syndrome, affected brain tissue must have contained both HY and DY agents since it retained mink pathogenicity. In competition experiments, we found that animals co-infected with the HY and DY agent strains produced only the HY syndrome. Other studies (Kimberlin & Walker, 1978a) have not observed blocking when two strains of the scrapie agent are injected at the same time.

Comparison of endpoint brain titres revealed that HY brain contained 100-fold more infectivity than DY brain. However, it is not likely that this large difference in infectivity is responsible for the differences in incubation period. HY brain diluted 10⁻⁷ produced incubation periods of 112 ± 2 days, well below the 168 days observed with a 10⁰% brain homogenate from DY brain. We are presently measuring the rate of growth for each strain during the course of infection. If the DY TME agent has a slower rate of growth, it may require a longer length of time to damage brain areas, or the clinical target area (CTA), responsible for causing clinical disease. Alternatively, HY- and DY-infected hamsters may have different CTAs since the diseases differ with respect to clinical signs and neuropathology. The severe spongiform degeneration in the central grey matter of the cerebellum in low passage HY TME and hamster scrapie (Marsh & Kimberlin, 1975) is a likely cause for the pronounced cerebellar ataxia in these two syndromes (Fulton, 1949). Yet animals affected with higher passage strains maintain the same predominant clinical signs while having only minimal cerebellar lesions. It would seem that still very little is known as to the relationship between agent titre, neuropathology and primary cell injury.

The HY and DY strains of agent replicated to a different brain titre in the same host, yet both caused disease. A previous study suggests that a critical threshold titre of agent is required before clinical disease develops (Kimberlin & Walker, 1978b). In mouse and hamster scrapie models, for a given host genotype and route of infection, no significant differences (i.e. >10-fold) in endpoint brain titres have been observed between different strains of the agent (Dickinson & Meikle, 1969; Kimberlin & Walker, 1977, 1978b; Carp & Callahan, 1986). Our studies are the first to demonstrate significant agent strain differences in brain titres at clinical disease when the host genotype and route of infection are kept constant.

Transmission of the Stetsonville source of TME to outbred Syrian golden hamsters resulted in host selection of the HY and DY agent strains. The host genes Sinc (Dickinson et al., 1968) or Prn-i (Carlson et al., 1986) that control the length of the scrapie incubation period in inbred strains of mice have not been demonstrated to play a role in host selection of strains in Syrian golden hamsters. However, differences in the primary amino acid sequence of the prion protein among different species of hamsters has been proposed to account for the observed differences in incubation periods and neuropathology (Lowenstein et al., 1990). In our study, the host genotype remains constant and two TME strains retain their characteristics after many serial passages. These differences in biological properties must lie within the informational molecule of each strain and not the host genotype. A previous study (Carlson et al., 1989) describes differences in the primary amino acid sequence of mouse donor prion proteins that are purported to be responsible for prion isolates with different biological properties. Our study and others (Dickinson et al., 1968; Bruce & Dickinson, 1987; Kimberlin et al., 1989) illustrate that strain differences can be maintained in the same host after several serial passages and, therefore, amino acid heterogeneity of the prion protein is not an absolute requirement for strain variation.

We have demonstrated that the HY and DY TME strains can be separated by host selection from a mixture. Whether the host is selecting either variants of the prion protein that can 'encode' strain-specific characteristics or a nucleic acid with virion-like properties remains
to be determined. We are presently analysing prion proteins from the two TME syndromes to determine whether differences can be detected.

We would like to thank Bruce Chesebro for use of animal facilities at the Rocky Mountain Laboratory, Hamilton, Montana, where some passage levels were performed in parallel to those done in Madison. These studies were supported by the College of Agricultural and Life Sciences, University of Wisconsin-Madison, and by a Biotechnology Grant, 87-CR-CR-1-2457, from the United States Department of Agriculture.

References


(Received 19 August 1991; Accepted 1 October 1991)