Journal of General Virology (2004), **85**, 265–273 DOI 10.1099/vir.0.19394-0

Delay in onset of prion disease for the HY strain of transmissible mink encephalopathy as a result of prior peripheral inoculation with the replication-deficient DY strain

Jason C. Bartz,[1] Judd M. Aiken[2] and Richard A. Bessen[1]†

[1]Department of Medical Microbiology and Immunology, Creighton University, Omaha, NE 68178, USA

[2]Department of Animal Health and Biomedical Sciences, University of Wisconsin, Madison, WI 53706, USA

Correspondence
Richard Bessen
rbessen@montana.edu

We report that the replication-deficient DY strain of transmissible mink encephalopathy (TME) can delay disease caused by the pathogenic HY TME strain. In this study, competition between the HY and DY TME agents was investigated following superinfection of the sciatic nerve and peritoneal cavity. Initially, DY TME infection was examined in the absence of superinfection and it was found that inoculation into the brain and sciatic nerve resulted in prion disease and PrP^{Sc} deposition in brain but not lymphoreticular tissues. Conversely, intraperitoneal inoculation of the DY TME agent did not result in clinical symptoms, DY TME agent replication or PrP^{Sc} deposition 400–600 days after infection. These findings indicate that the DY TME agent does not replicate in secondary lymphoid organs and is non-pathogenic when neuroinvasion is dependent on prior infection of the lymphoreticular system. However, intraperitoneal inoculation of the DY TME agent at 60 days, but not at 30 days, prior to intraperitoneal inoculation of the HY TME agent resulted in an extension of the HY TME incubation period. Inoculation of the DY TME agent into the sciatic nerve at 60 days prior to intrasciatic nerve inoculation of the HY TME agent did not delay the incubation period of HY TME. The ability of the DY TME agent to delay HY TME infection following extraneural inoculation, but not neural infection, suggests that HY and DY TME agent competition can occur in a common replication site whose cellular location precedes infection of both the lymphoreticular and peripheral nervous systems.

Received 4 June 2003
Accepted 1 October 2003

INTRODUCTION

Prion diseases are a group of fatal neurodegenerative disorders of humans and animals that can have either an infectious, inherited or sporadic aetiology. A central feature of these diseases is the conversion of the host prion protein gene product, PrP^{C}, to a misfolded conformation, PrP^{Sc} (Prusiner, 1991). This post-translational modification of PrP^{C} into PrP^{Sc} is associated with replication of the infectious prion agent and there is strong evidence to support the proposal that PrP^{Sc} is the only component of the agent. Among the prion diseases, there exist distinct disease phenotypes caused by different prion strains. These prion strains can differ with respect to the biochemical and physical properties of PrP^{Sc}, the incubation period, clinical symptoms and neuropathology when introduced into a common host species.

In flocks of sheep with endemic scrapie or in free-ranging deer populations with a high incidence of chronic wasting disease, there is an increased opportunity for exposure of an individual animal to multiple prion strains. Experimental infection of mice with two strains of scrapie at separate times (i.e. superinfection) by intraperitoneal (i.p.) inoculation can result in murine scrapie strain 22A (a long-incubation-period strain) blocking disease caused by scrapie strain 22C (a short-incubation-period strain) (Dickinson *et al.*, 1972, 1975). As a result, mice die from disease caused by 22A scrapie and there is no evidence of 22C scrapie infection in the brain. The basis for this blocking effect is proposed to be due to 22A scrapie replication in a majority of the limited number of replication sites in the spleen and other secondary lymphoid tissues, and as a result 22C scrapie infection is prevented upon superinfection (Dickinson & Fraser, 1979; Dickinson *et al.*, 1975). This model is supported by reports that demonstrate that the outcome of superinfection depends upon the dose of the inoculated prion strains and the time interval separating the two

†Present address: Department of Veterinary Molecular Biology, PO Box 173610, Montana State University, Bozeman, MT 59717, USA.

infections. Greater blocking effects are achieved by either increasing the dose of 22A scrapie or by increasing the time interval before inoculation of 22C scrapie (Dickinson & Fraser, 1979), indicating that this effect is mediated by a higher amount or longer period of 22A scrapie replication prior to superinfection. The ability of 22A scrapie to block 22C scrapie following extraneural infection is also dependent on inoculation of a dose of 22A that results in clinical disease, since this effect is lost upon prior heat or chemical inactivation of 22A scrapie (Kimberlin & Walker, 1985). Both a delay and a blocking effect on a fast prion strain by a slow prion strain have been reported following superinfection by the intracerebral (i.c.) route of inoculation (Dickinson *et al.*, 1972; Manuelidis, 1998). In the former case, mice inoculated with 22A scrapie agent died from a subsequent 22C scrapie superinfection but the time to onset of disease was protracted compared with a control 22C scrapie infection (Dickinson *et al.*, 1972).

Experimental i.c. co-infection (i.e. simultaneous inoculation) with the HY (a short-incubation-period strain) and DY (a long-incubation-period strain) strains of transmissible mink encephalopathy (TME) illustrates that the DY TME agent can block HY TME disease, and as a result hamsters develop clinical signs of DY TME (Bartz *et al.*, 2000). Subsequent serial passage of brain tissue from co-infected animals exhibiting DY TME disease can result in either the emergence of HY TME disease or maintenance of the DY TME phenotype, depending on the doses used during the initial co-infection. In the former case, both TME strains are able to replicate during co-infection, but upon serial passage, the dose of the short incubation HY TME agent becomes greater than it was during the initial co-infection and is responsible for causing clinical disease. The strain-specific PrP^{Sc} pattern also switches from that of the DY PrP^{Sc} conformation to that of the HY PrP^{Sc} conformation during the transition from clinical symptoms of DY TME to HY TME upon serial prion passage (Bartz *et al.*, 2000). The blocking effects of DY TME co-infection on HY TME are only observed following i.c. inoculation and not by co-infection by the i.p. route. This latter finding is most likely due to the inability of the DY TME agent to cause clinical disease more than 300 days post-infection following i.p. inoculation (Bessen & Marsh, 1992b).

In the current study, we investigated the ability of the DY TME agent to either delay or block HY TME infection following superinfection by intrasciatic nerve (i.n.) and i.p. routes of inoculation. We have demonstrated that prior inoculation of the DY TME agent can delay the onset of HY TME disease following i.p. inoculation, despite the inability of the DY TME agent to replicate in the lymphoreticular and nervous system tissues by this route of inoculation. Conversely, inoculation of the DY TME agent into the sciatic nerve did not delay HY TME disease upon superinfection, even though the DY TME agent can replicate and cause disease in the absence of superinfection. These findings suggest that either (i) the DY TME agent can replicate at locations other than the secondary lymphoid organs and nervous system following i.p. inoculation and that competition with the HY TME agent at these sites upon extraneural superinfection results in a delay in the progression of HY TME infection; or (ii) low levels of DY TME replication in the lymphoreticular system or nervous system, which cannot be detected by animal bioassay, are able to delay HY TME disease upon superinfection.

METHODS

Animal inoculations and tissue collection. All procedures involving animals were approved by the Creighton University IACUC and comply with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996). Weanling (4–5-week-old), outbred Golden Syrian hamsters (Harlan Sprague–Dawley, IN, USA) were i.c. inoculated with 25 µl of a 1 % (w/v) brain homogenate from an HY TME- or DY TME-infected hamster containing $10^{7·5}$ or $10^{5·4}$ medial lethal doses (LD_{50}) ml^{-1}, respectively. Alternatively, 4–5-week-old hamsters were i.p. inoculated with 100 µl of a 1 % (w/v) brain homogenate from an HY TME-infected or DY TME-infected hamster. For inoculation of the sciatic nerve, minor surgery was performed as previously described (Bartz *et al.*, 2002). Animals were observed three times per week for the onset of neurological disease, as previously described (Bartz *et al.*, 2002). Hamsters were killed by CO_2 asphyxiation and tissues were removed for analysis as indicated below.

For the TME superinfection studies described in Fig. 2, DY TME brain homogenates were inoculated either at 30 or 60 days prior to the HY TME agent inoculation, and the incubation period was measured from the time of superinfection. The dose of the TME agent inoculated is given in i.c. LD_{50}. Inoculation of a normal brain homogenate at 60 days prior to the HY TME agent inoculation was used as a control.

Bioassay for DY TME infectivity. Tissues (i.e. brain, spleen and sympathetic chain) were aseptically collected from hamsters at various time points following i.p. inoculation with the DY TME agent. The tissues were minced with disposable razor blades and sterile saline was added to a final volume of 250 µl for the spleen and sympathetic chain, or to a volume to make a 10 % (w/v) brain homogenate. The tissue was homogenized using a 26-gauge needle prior to sonication in a cup horn sonicator (Fisher Scientific). Tissue homogenates were i.c. inoculated into Syrian hamsters and the time to onset of clinical symptoms was measured. The entire volume of the spleen and sympathetic chain homogenates were each inoculated into a total of six hamsters.

Tissue preparation and Western blot of PrP^{Sc}. For PrP^{Sc} analysis of brain from clinically ill hamsters, a 5 % (w/v) homogenate in Dulbecco's PBS without Ca^{2+} or Mg^{2+} (Mediatech) was digested with 0·4 U proteinase K ml^{-1} (Roche Diagnostics). Homogenates were incubated at 37 °C for 1 h with constant agitation followed by the addition of PMSF to a concentration of 5 mM. Proteinase K-digested brain homogenates (0·25 mg equivalents) were analysed for PrP^{Sc} content by SDS-PAGE and Western blotting as described below.

The spleen and medial iliac lymph node were homogenized in 10 mM Tris/HCl, pH 7·5, containing 5 mM $MgCl_2$ to produce a 20 % (w/v) tissue suspension. Brain from hamsters that did not develop clinical symptoms was homogenized in PBS containing 5 mM $MgCl_2$ to make a 10 % (w/v) homogenate. Tissue homogenates were incubated with 100 U Benzonase nuclease ml^{-1} (Novagen) at 37 °C for 1 h with constant agitation. An equal volume of buffer A (10 mM Tris/HCl, pH 7·5, 1 mM dithiothreitol) containing 20 % (w/v) *N*-lauroylsarcosine

was added and the samples were mixed on a Vortex Genie (VWR Scientific) for 30 min at room temperature. Enrichment for PrP^{Sc} and proteinase K digestion were performed as previously described (Bartz *et al.*, 2002). SDS-PAGE and Western blotting were performed as previously described (Bartz *et al.*, 2002; Bessen & Marsh, 1994) using monoclonal anti-PrP antibody 3F4 ascites fluid at a 1 : 40 000 dilution (a gift from R. Kascsak, New York State Institute for Basic Research in Developmental Disabilities, Staten Island, NY, USA) or 3F4 hybridoma supernatant at a 1 : 10 000 dilution (a gift from V. Lawson, National Institute of Allergy and Infectious Diseases, Rocky Mountain Laboratories, Hamilton, MT, USA) (Kascsak *et al.*, 1987).

Statistical analysis. Incubation period data from hamsters infected with TME agents were compared using SigmaStat 2.0 software (SPSS Inc.). The *t*-test (paired) or the non-parametric Mann–Whitney U-test was used depending on whether the data had a normal distribution pattern or not. In all comparisons, a P value less than 0·01 was used to determine whether two datasets were statistically different (Fig. 2).

RESULTS

Pathogenicity of HY and DY TME following neural and extraneural routes of inoculation

To investigate the ability of the HY and DY TME agents to cause disease following inoculation of the central nervous system, peripheral nervous system and an extraneural route of inoculation, hamsters were inoculated by the i.c., i.n. and i.p. routes. Golden Syrian hamsters inoculated with $10^{5·9}$ LD_{50} of the HY TME agent or $10^{3·8}$ LD_{50} of the DY TME agent by the i.c. route resulted in an incubation period of 60 ± 2 (10/10) days and 168 ± 5 (5/5) days (days $\pm$ SEM, with the number affected/number inoculated indicated in parentheses), respectively (Table 1). Clinical symptoms of HY TME included ataxia and hyperexcitability and these signs were clearly distinguishable from DY TME, which was characterized by a progressive lethargy as previously described (Bessen & Marsh, 1992b). The different incubation periods between HY TME and DY TME were not due to the 100-fold larger dose of the HY TME agent used since i.c. inoculation of $10^{3·9}$ LD_{50} of the HY TME agent resulted in an incubation period that was approximately 100 days shorter than the incubation period following inoculation of $10^{3·8}$ LD_{50} of the DY TME agent (data not shown). The incubation periods for the HY TME ($10^{5·2}$ LD_{50}) and DY TME ($10^{3·1}$ LD_{50}) agents were longer following inoculation of the sciatic nerve compared with i.c. inoculation ($P < 0·01$, *t*-test and Mann–Whitney U-test); the onset of clinical symptoms was at 69 ± 2 (6/6) days and 210 ± 9 (5/6) days, respectively (Table 1). I.p. inoculation of the HY TME agent ($10^{7·5}$ LD_{50}) had an incubation period of 130 ± 4 (5/6) days, but following i.p. inoculation of the DY TME agent ($10^{4·4}$ LD_{50}), none of the hamsters had developed symptoms of DY TME by 600 days post-infection (Table 1). At 405 days post-infection, one hamster that was i.p. inoculated with the DY TME agent died of unknown causes and did not exhibit symptoms of TME. These findings indicate that the DY TME agent can cause disease when inoculated into the peripheral and central nervous systems but does not result in clinical TME following inoculation into the peritoneal cavity.

The accumulation of the HY and DY TME agents in the lymphoreticular system (LRS) and brain of TME-infected hamsters inoculated by each route of inoculation was investigated by PrP^{Sc} Western blotting. HY and DY PrP^{Sc} have a different polypeptide pattern by Western blotting: the HY PrP^{Sc} polypeptides are approximately 2 kDa larger than the corresponding polypeptides from DY PrP^{Sc}, as previously reported (Bessen & Marsh, 1992a, 1994). For HY TME, the lowest molecular mass PrP^{Sc} polypeptide was 21 kDa, while for DY PrP^{Sc} it migrated at 19 kDa (Fig. 1). In HY TME-inoculated hamsters, PrP^{Sc} was found in the brain at terminal disease for the i.c., i.p. and i.n routes of inoculation and was present in the spleen and lymph nodes following i.c. and i.p. inoculation but not i.n. inoculation (Fig. 1). The 21 kDa HY TME strain-specific PrP^{Sc} polypeptide pattern was found in each of these cases. For DY TME infection, the 19 kDa strain-specific PrP^{Sc} pattern was present in the brain in clinically ill hamsters but was absent from the spleen and medial iliac lymph node, regardless of the inoculation route and health status of the animal. The absence of PrP^{Sc} in the lymph nodes (e.g. mesenteric, medial iliac and submandibular) of DY TME infected hamsters was confirmed by PrP immunohistochemistry (data not shown). Clinically normal hamsters killed at 80 days, 184 days and more than 500 days post-infection following i.p. inoculation also had no evidence of PrP^{Sc} deposition in the LRS and brain (Fig. 1 and data not shown). The absence of DY PrP^{Sc} in lymphoreticular tissues suggests that PrP^{Sc} formation

Table 1. Incubation period of HY TME and DY TME following neural and extraneural routes of inoculation

Route	Incubation period of HY TME*	A/I†	Incubation period of DY TME*	A/I†
Cerebral	60 ± 2	10/10	168 ± 5	5/5
Sciatic nerve	69 ± 2	6/6	210 ± 9	5/6
Peritoneal	130 ± 4	5/6	>600	0/12‡

*Days $\pm$ SEM to the onset of clinical signs.

†Number affected/number inoculated.

‡One intercurrent death at 405 days post-infection.

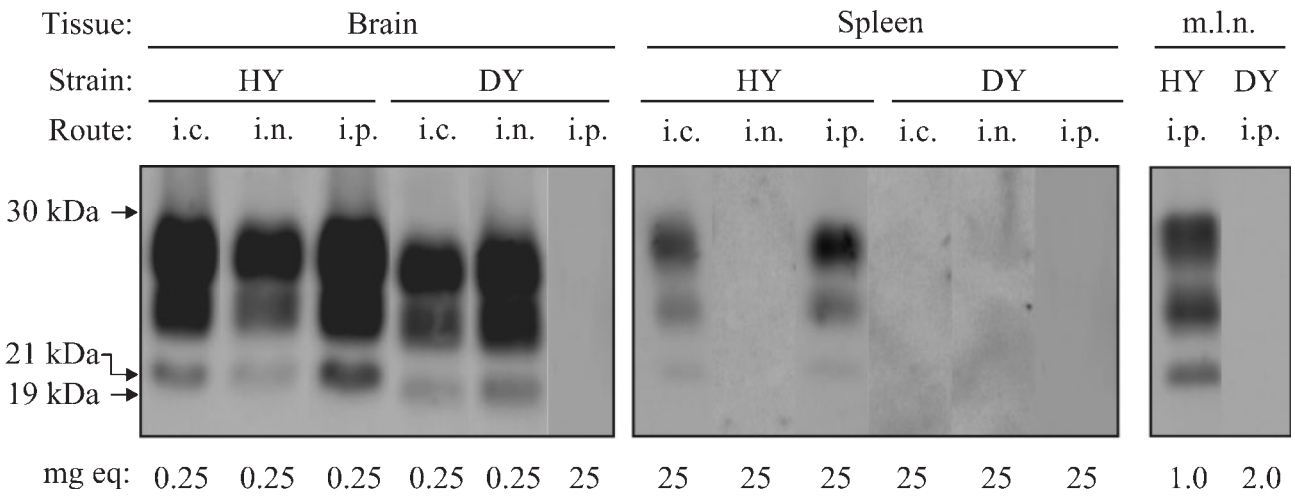

Fig. 1. Tissue distribution of PrP^{Sc} following inoculation of the HY TME and DY TME agents by neural and extraneural routes. Hamsters were inoculated with the TME agent by either the intracerebral (i.c.), intraperitoneal (i.p.) or sciatic nerve (i.n.) route. Tissue was collected from animals that had been killed after the onset of clinical disease and from clinically normal, age-matched DY TME-inoculated hamsters at 184 days (medial iliac lymph node) and at more than 500 days (brain and spleen) after i.p. inoculation. The brain, spleen and medial iliac lymph nodes (m.l.n) were collected for PrP^{Sc} analysis. Tissue homogenates, containing 0·25 mg equivalents of tissue (mg eq) and PrP^{Sc}-enriched preparations (>0·25 mg eq) were prepared as described in the text and analysed by SDS-PAGE and PrP Western blotting. Molecular mass markers (kDa) are shown on the left of the panel and the position of the lower molecular mass HY TME PrP^{Sc}-specific band at 21 kDa and the DY TME PrP^{Sc}-specific band at 19 kDa are indicated.

either does not occur in these tissues or is below the limit of detection by Western blotting (i.e. <1000 LD_{50}).

DY TME replication following intraperitoneal inoculation

To determine whether low levels of the DY TME agent were present in tissues that were PrP^{Sc} negative following i.p. inoculation, the amount of infectivity was measured in the entire spleen and sympathetic chain at 80 days and 184 days post-infection by animal bioassay. Hamsters inoculated with tissue homogenates did not develop DY TME by day 365 post-infection (Table 2) suggesting that the DY TME agent did not replicate in the spleen and sympathetic chain of hamsters for up to 6 months. After 365 days, hamsters inoculated with spleen or sympathetic chain homogenates were killed and the brains were analysed for PrP^{Sc} by Western blotting but PrP^{Sc} was not found (data not shown). The DY TME agent was also not detected by animal bioassay (Table 2) or by PrP^{Sc} Western blotting (data not shown) in a brain homogenate from a hamster that died from unknown causes at day 405 post-infection following i.p. inoculation. A control DY TME brain homogenate containing $10^{1·8}$ LD_{50} caused disease at 210 ± 7 days (4/4) when i.c. inoculated into hamsters (data not shown). These animal bioassay studies demonstrated that less than 100 LD_{50} of the DY TME agent was present in the spleen and autonomic and central nervous systems of hamsters following i.p.

Table 2. TME infectivity in tissues from hamsters intraperitoneally inoculated with the DY TME agent

DY TME (days post-inoculation)*	Incubation period for spleen (days)†	A/I‡	Incubation period for sympathetic chain (days)†	A/I‡	Incubation period for brain (days)†	A/I‡
80	>365	0/6	>365	0/6	ND	ND
184	>365	0/6	>365	0/6	ND	ND
405	ND	ND	ND	ND	>600	(0/9)

*Hamsters were i.p. inoculated with $10^{4·4}$ median lethal doses of the DY TME agent and the spleen, sympathetic chain and brain were collected at the indicated times post-inoculation for animal bioassay.

†Days ± SEM to the onset of clinical signs.

‡Number affected/number inoculated.

ND, Not determined.

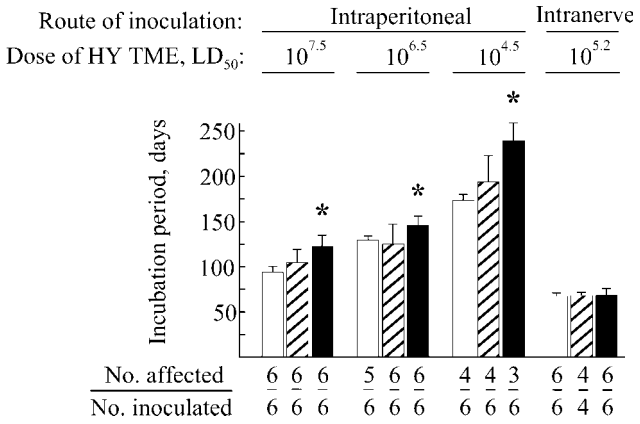

Fig. 2. TME incubation period following superinfection of hamsters with the HY TME and DY TME agents. The DY TME agent was inoculated into the peritoneal cavity ($10^{4\cdot4}$ LD_{50}) or sciatic nerve ($10^{3\cdot1}$ LD_{50}) at 30 days (hatched bars) or at 60 days (solid bars) prior to inoculation of the HY TME agent. The TME incubation period was measured in days post-inoculation with the HY TME agent. A control group was inoculated with the HY TME agent alone (open bars). The amount of the HY TME agent inoculated (LD_{50}) and the number of affected and inoculated hamsters are indicated. Asterisks denote incubation periods in superinfected hamsters that were significantly different (*t*-test or Mann-Whitney U-test, $P<0\cdot01$) from control hamsters inoculated with the HY TME agent (open bars).

inoculation. This was consistent with the absence of PrP^{Sc} in the spleen, medial iliac lymph node and brain by Western blotting (Fig. 1). The lack of DY TME infectivity and PrP^{Sc} in spleen following inoculation of the DY TME agent by neural and extraneural routes suggests that the DY TME agent cannot replicate in the LRS. These findings also indicate that levels of the DY TME agent that could be below the threshold of experimental detection in the spleen did not spread to the autonomic nervous system and brain, where higher levels of DY TME agent replication can occur.

Superinfection of DY TME-inoculated hamsters with HY TME by the intraperitoneal route

To investigate the ability of the DY TME agent to delay the incubation period of the HY TME agent following i.p. infection, hamsters were i.p. inoculated with the DY TME agent ($10^{4\cdot4}$ i.c. LD_{50}) at either 30 days or 60 days prior to i.p. inoculation of the HY TME agent in three different doses (i.e. $10^{7\cdot5}$, $10^{6\cdot5}$, or $10^{4\cdot5}$ i.c. LD_{50}). In the control groups that received only a single i.p. inoculation of the HY TME agent, the incubation period for each of these doses was 94 ± 6 (6 affected/6 inoculated), 130 ± 4 (5/6) and 174 ± 4 (4/6) days, respectively (Fig. 2, open bars). (Doses of the HY TME agent at or below $10^{4\cdot5}$ i.c. LD_{50} do not always cause disease following i.p. inoculation (Fig. 2); therefore, 100-fold and 1000-fold higher doses were also included in these experiments in order to reproducibly establish HY TME disease.) I.p. inoculation of the DY TME agent at 30 days prior to i.p. inoculation of the three doses of the HY TME agent resulted in clinical signs of HY TME at 104 ± 18 (6/6), 126 ± 23 (6/6) and 193 ± 30 (4/6) days post-infection (Fig. 2, hatched bars). These incubation periods were not statistically different ($P>0\cdot05$, *t*-test and Mann–Whitney U-test) from the control groups that were inoculated only with the HY TME agent, despite the appearance of an upward trend in incubation period. Inoculation of the DY TME agent at 60 days prior to i.p. inoculation of the HY TME agent resulted in the onset of clinical signs of HY TME at 123 ± 13 (6/6), 146 ± 11 (6/6) and 239 ± 22 (3/6) days post-infection, respectively (Fig. 2, solid bars). The incubation periods for the three groups that each received different doses of the HY TME agent at 60 days after inoculation with the DY TME agent were statistically different ($P<0\cdot01$; *t*-test or Mann–Whitney U-test) from the control groups that received a similar dose of the HY TME agent without prior infection with the DY TME agent, but they were not statistically different ($P>0\cdot05$) from hamsters that were superinfected with the HY TME agent at 30 days after inoculation with the DY TME agent. I.p. inoculation of a 1 % (w/v) normal brain homogenate at 60 days prior to i.p. inoculation of the HY TME agent at $10^{6\cdot5}$ LD_{50} resulted in an incubation period of 123 ± 4 days (6/6) (data not shown). This value was also statistically different ($P<0\cdot01$; Mann–Whitney U-test) from the age-matched hamster group that first received the DY TME agent at 60 days prior to the same dose of the HY TME agent (i.e. 146 ± 11 days; Fig. 2). These findings indicate that i.p. inoculation of the DY TME agent at 60 days prior to i.p. inoculation of the HY TME agent can delay the onset of HY TME symptoms.

In the TME superinfection experiment (Fig. 2), all hamsters that developed TME had a clinical phenotype characteristic of HY TME and also had a 21 kDa PrP^{Sc} polypeptide pattern that was characteristic of the HY TME strain (Fig. 3). None of the hamsters exhibited symptoms of DY TME or had evidence of the 19 kDa PrP^{Sc} polypeptide pattern in the brain that is typical for DY TME infection (Fig. 3). These results indicated that, following superinfection with the DY and HY TME agents by i.p. inoculation, hamsters acquired an HY TME infection in the brain and this strain was responsible for causing TME.

Superinfection of DY TME-inoculated hamsters with the HY TME agent by the intranerve route

We next examined the ability of DY TME infection to delay or block HY TME infection in the peripheral nervous system by inoculating the sciatic nerve with the DY TME agent prior to i.n. inoculation with the HY TME agent. Hamsters were inoculated in the right sciatic nerve with the DY TME agent ($10^{3\cdot1}$ i.c. LD_{50}) at 30 or 60 days prior to inoculation of the HY TME agent ($10^{5\cdot2}$ i.c. LD_{50}). The incubation period for these groups was 69 ± 2 (4/4) and 69 ± 8 (6/6) days, respectively, which was not statistically different ($P>0\cdot05$; *t*-test) from animals that only received an i.n. inoculation of the HY TME agent (67 ± 2 days; 6/6) (Fig. 2). All hamsters exhibited clinical symptoms of HY TME and had a 21 kDa

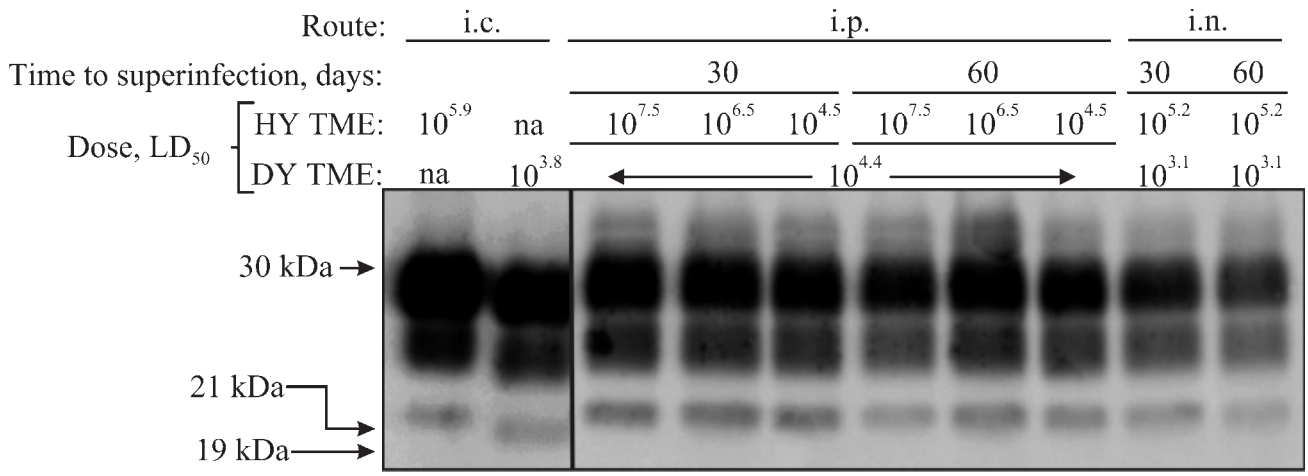

Fig. 3. PrP^{Sc} deposition in the brains of hamsters following infection with the DY TME and HY TME agents. Animals were inoculated by the intraperitoneal (i.p.) or sciatic nerve (i.n.) routes with the DY TME agent at 30 or 60 days prior to inoculation with the HY TME agent. Control hamsters were inoculated by the intracerebral route (i.c.) with either the HY TME or the DY TME agent. Each lane contains 0·25 mg equivalents of proteinase K-digested brain homogenate. PrP^{Sc} analysis was performed as described in Fig. 1. The amount of inoculum is given as LD_{50} and, for i.c. inoculated hamsters, TME superinfection is not applicable (na). Molecular mass markers (kDa) are shown on the left of the panel and the position of the lower molecular mass HY TME PrP^{Sc}-specific band at 21 kDa and the DY TME PrP^{Sc}-specific band at 19 kDa are indicated.

PrP^{Sc} polypeptide migration pattern in the brain that was characteristic of HY TME infection (Fig. 3). These findings indicated that prior establishment of DY TME infection in the sciatic nerve did not delay the development of HY TME disease following sciatic nerve superinfection.

In the absence of HY TME superinfection, DY TME infection of the sciatic nerve resulted in PrP^{Sc} deposition in the vertebral segments of the thoracic spinal cord (corresponding to the lumbar to mid-thoracic spinal cord segments) at 90 days p.i., but not at 60 day p.i., indicating that the DY TME agent entered the spinal cord from the sciatic nerve between 60 and 90 days p.i. (Fig. 4A). Following HY TME infection of the sciatic nerve, in the absence of prior DY TME infection, HY PrP^{Sc} was found in vertebral thoracic spinal cord segments 10–13 at 28 days post-infection (Fig. 4B). Therefore, the earliest time point that HY PrP^{Sc} could reach the spinal cord when i.n. inoculated at 60 days after the DY TME agent would be 88 days after the initial DY TME agent inoculation (60 + 28 days). The presence of DY PrP^{Sc} in the thoracic and lumbar spinal cord at the time of HY PrP^{Sc} entry into the lumbar spinal cord was not sufficient to delay or block the onset of HY TME following superinfection of the sciatic nerve.

DISCUSSION

In this study we have demonstrated that the DY TME agent can cause disease following inoculation into the peripheral and central nervous systems but that the DY TME agent is unable to replicate in the LRS during infection. Direct inoculation of the DY TME agent into the peritoneal cavity did not result in (i) PrP^{Sc} accumulation in the spleen, lymph nodes and brain; (ii) TME replication in the spleen and sympathetic nervous system; or (iii) clinical TME. These findings demonstrate that TME amplification in the LRS and subsequent neuroinvasion were absent following extraneural inoculation of the DY TME agent. This is the first report of a pathogenic prion strain that cannot establish infection in the LRS upon intraspecies transmission. In murine scrapie, i.p. inoculation with the 87V strain of the scrapie agent can establish a subclinical scrapie infection for the lifespan of the host, but in this case, 87V agent replication is found in the spleen within weeks of infection and in the brain at later times post-infection (Bruce, 1985; Collis & Kimberlin, 1985). In the current study, a TME bioassay of entire spleens at 80 and 184 days after i.p. inoculation of hamsters with the DY TME agent did not result in detectable infectivity, indicating that either the DY TME agent was not transported to the spleen, does not replicate in the spleen, or the level of replication was below the limit of detection for the animal bioassay (i.e. less than 100 LD_{50} per spleen). If low levels of DY TME infection were present in the spleen at 80 days p.i., it is anticipated that by 600 days p.i., the DY TME agent would spread to the autonomic and central nervous systems and replicate to higher levels than found in the spleen, similar to a subclinical 87V scrapie infection in mice. However, at 405 days p.i. there was no DY TME infectivity found in the brain and no PrP^{Sc} deposition in the brain at more than 500 days p.i. These findings strongly suggest that the DY TME agent does not replicate in LRS tissues or spread to the nervous system following extraneural inoculation. These findings have

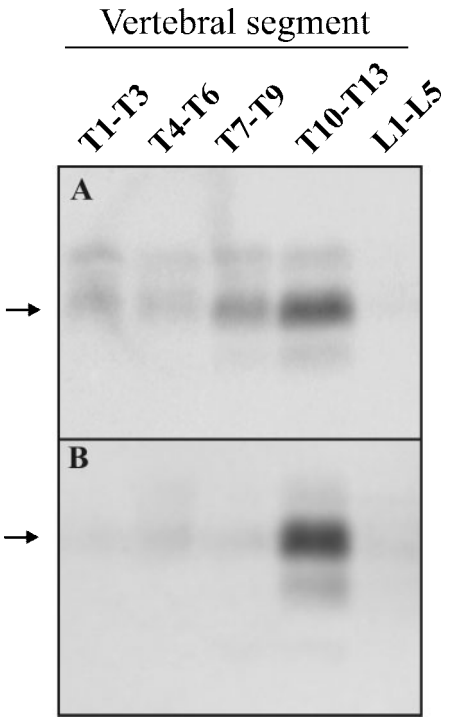

Fig. 4. PrP^{Sc} deposition in the spinal cord of hamsters following sciatic nerve infection with the DY TME and HY TME agents. Animals were inoculated into the sciatic nerve with the DY TME or HY TME agents, and at monthly or weekly intervals, the spinal cord was collected and dissected into thoracic (T) and lumbar (L) vertebral segments (indicated by numbers). PrP^{Sc} Western blot analysis was performed on 0·50 mg tissue equivalents of proteinase K-digested spinal cord homogenates from a DY TME-infected hamster at 90 days post-infection (A) and a HY TME-infected hamster at 28 days post-infection (B). The arrow at the left of the panel indicates the highest molecular mass PrP^{Sc} polypeptide band. The immunoreactivity above the arrow was consistently found in uninfected spinal cord homogenates and most likely represents non-specific immunoreactivity.

parallels in natural BSE in which PrP^{Sc} and infectivity have not been found in the LRS, even though both are present in the peripheral and central nervous systems (Somerville *et al.*, 1997; Wells *et al.*, 1998). It is possible that the BSE agent can directly enter the autonomic nervous system without prior infection of the LRS following oral ingestion (McBride *et al.*, 2001; Schulz-Schaeffer *et al.*, 2000). However, low levels of BSE infectivity were recently found in a single tonsil and in the distal ileum following experimental oral BSE infection (Spongiform Encephalopathy Advisory Council, 2001; Terry *et al.*, 2003). These findings suggest that very low levels of the BSE agent could be present in the LRS of cattle. Perhaps in DY TME infection, there is also a low level or transient infection of the LRS.

These studies also demonstrate that the LRS replication-deficient DY TME agent can delay the onset of HY TME following HY TME agent superinfection by the i.p. route, but not by an i.n. route of inoculation. In previous studies with murine-adapted scrapie and Creutzfeldt–Jakob disease prions, the long-incubation-period prion strains could delay or block the short-incubation-period strains only if they were able to cause disease in the absence of superinfection (Dickinson *et al.*, 1972, 1975; Kimberlin & Walker, 1985; Manuelidis, 1998; Manuelidis & Yun Lu, 2000). The current findings are paradoxical, since the DY TME agent does not cause TME or replicate in the LRS and nervous system following i.p. inoculation, yet it was able to delay HY TME following i.p. superinfection. An explanation for these findings could be that the DY TME brain inoculum induced an immune response to host components in the inoculum or to the DY TME agent, which is cross-reactive with the HY TME agent. The former outcome is unlikely since a normal brain homogenate inoculated prior to the HY TME brain homogenate did not delay disease onset. Furthermore, there was no delay in the onset of HY TME following superinfection at 30 days after DY TME agent inoculation, indicating that an adaptive immune response to normal brain components did not delay HY TME infection. The production of an immune response to the DY TME agent that can affect HY TME infection is also unlikely because a more efficacious immune response would be expected during an infection in which the DY TME agent is replicating compared with a situation where no DY TME agent is found. However, under conditions in which the DY TME agent does not replicate in the LRS and nervous system (e.g. i.p. inoculation), there was a statistically significant delay in the HY TME incubation period, but in experiments where the DY TME agent could replicate (e.g. i.n. inoculation), there was no delay following HY TME superinfection. Therefore, DY TME agent replication does not correlate with an undefined protective anti-prion immune response or the ability to delay HY TME disease onset following superinfection. We cannot exclude the possibility that i.p. inoculation of the DY TME agent induces an immune response to the TME agent in the absence of LRS replication, but other studies (Garfin *et al.*, 1978; Kasper *et al.*, 1982; Kingsbury *et al.*, 1981; Porter *et al.*, 1973) have been unsuccessful in identifying a prion-specific immune response or antibodies to PrP^{Sc} following prion infection.

In the current study, we were unable to demonstrate TME agent strain competition in the peripheral and central nervous systems following i.n. inoculation. Our findings are different from previous ones (Dickinson *et al.*, 1972; Manuelidis, 1998), which demonstrate that a slow prion strain can delay or block a fast prion strain following i.c. inoculation, despite the presence of DY TME replication in the spinal cord prior to the spread of the HY TME agent into the cord from the sciatic nerve. One explanation could be that there are fewer common replication sites between the TME agent strains following i.n. compared with i.c. inoculation, especially if the HY and DY TME agents use different spinal tracts to ascend to the brain. We previously demonstrated that the HY TME agent can travel in the

retrograde direction within descending spinal motor tracts following i.n. inoculation (Bartz *et al.*, 2002) but the pathways used by the DY TME agent strain in the spinal cord are uncertain. Perhaps, if the DY TME agent is more dependent on transport within sensory spinal tracts to spread to the brain, then following superinfection with the HY TME agent, the fast TME strain can travel to the brain with minimal overlap of replication sites used by the DY TME agent. In this case, there would not be sufficient competition between the TME strains to result in a delay or block of HY TME infection. Another possible explanation for the absence of a delay in the HY TME incubation period following i.n. superinfection could be that the length of time to HY TME superinfection was too short and, as a result, there was insufficient DY TME agent replication to affect the subsequent HY TME infection. However, it is noteworthy that, in previous prion superinfection studies (Dickinson *et al.*, 1972; Manuelidis, 1998) in which a slow prion strain blocked a fast prion strain, 18–22 % of the incubation period of the slow prion strain elapsed before inoculation of the fast prion strain. In the present study, the HY TME agent was i.n. inoculated after 29 % of the anticipated DY TME incubation period by the i.n. route had elapsed (at day 60 of a 210 day incubation period). Therefore, a proportionally longer time period for DY TME agent replication was permitted in the sciatic nerve and/or spinal cord prior to HY TME agent superinfection but competition between the TME agent strains was not found.

The mechanism by which the DY TME agent delays the onset of clinical signs of HY TME following superinfection by the i.p. route could be dependent on replication of the two TME agent strains in a common tissue replication site. In the current study, both TME strains can replicate in the nervous system but this site is unlikely to be the location of agent strain competition since (i) there was no evidence of DY TME agent replication in the peripheral and central nervous systems following i.p. inoculation; and (ii) superinfection by the i.n. route did not result in a delay in HY TME disease onset, even though DY TME agent replication in the spinal cord preceded HY TME agent spread to the spinal cord. One possible site for TME agent strain competition following i.p. superinfection is in the LRS, even though we were unable to detect PrP^{Sc} deposition or DY TME infectivity in secondary lymphoid tissues. In this scenario, low levels of DY TME agent replication may occur in the lymph nodes since we did not directly assay for DY TME infectivity at this site. DY TME replication in lymph nodes could partially block access of the HY TME agent to replication sites and/or delay subsequent HY TME infection of peripheral nerves, which is necessary for spread to the brain. We also noticed that in HY TME-infected hamsters, the PrP^{Sc} load per weight of tissue was greater in the medial iliac lymph node than in the spleen and, although we were unable to detect PrP^{Sc} in the lymph nodes of DY TME-infected hamsters, the small size of these tissues restricted PrP^{Sc} analysis. It is also noteworthy that in Golden Syrian hamsters infected with the 263K scrapie strain, splenectomy does not delay the onset of clinical disease following extraneural inoculation (Kimberlin & Walker, 1977, 1986), suggesting that other secondary lymphoid tissues are sufficient to support scrapie agent replication prior to neuroinvasion. Studies with immunodeficient mice have also demonstrated that scrapie infection of lymph nodes can occur in the absence of spleen infection (Prinz *et al.*, 2002), suggesting that within the LRS there are differences in prion targeting. Currently, we are examining lymph nodes in DY TME-infected hamsters for evidence of prion infection. Another potential explanation for our extraneural TME superinfection studies is that TME agent strain competition can occur at a site whose location precedes infection of both the LRS and nervous system. Peritoneal macrophages or other macrophage subsets are potential targets of TME agent replication that could play a role in the early pathogenesis of prion diseases.

ACKNOWLEDGEMENTS

This work was supported by US Department of Agriculture grant number 2002-35204-12584 (R. A. B.) and USDA post-doctoral fellowship number 00-35204-9228 (J. C. B.) from the National Research Initiative Competitive Grants Program. Special thanks to Emily Hansen for excellent technical assistance, Anthony E. Kincaid and Debbie I. McKenzie for critical reading of this manuscript and Matt Irma Caniglia for helpful discussions.

REFERENCES

Bartz, J. C., Bessen, R. A., McKenzie, D., Marsh, R. F. & Aiken, J. M. (2000). Adaptation and selection of prion protein strain conformations following interspecies transmission of transmissible mink encephalopathy. *J Virol* **74**, 5542–5547.

Bartz, J. C., Kincaid, A. E. & Bessen, R. A. (2002). Retrograde transport of transmissible mink encephalopathy within descending motor tracts. *J Virol* **76**, 5759–5768.

Bessen, R. A. & Marsh, R. F. (1992a). Biochemical and physical properties of the prion protein from two strains of the transmissible mink encephalopathy agent. *J Virol* **66**, 2096–2101.

Bessen, R. A. & Marsh, R. F. (1992b). Identification of two biologically distinct strains of transmissible mink encephalopathy in hamsters. *J Gen Virol* **73**, 329–334.

Bessen, R. A. & Marsh, R. F. (1994). Distinct PrP properties suggest the molecular basis of strain variation in transmissible mink encephalopathy. *J Virol* **68**, 7859–7868.

Bruce, M. E. (1985). Agent replication dynamics in a long incubation period model of mouse scrapie. *J Gen Virol* **66**, 2517–2522.

Collis, S. C. & Kimberlin, R. H. (1985). Long-term persistence of scrapie infection in mouse spleens in the absence of clinical disease. *FEMS Microbiology Lett* **29**, 111–114.

Dickinson, A. G. & Fraser, H. (1979). The scrapie replication-site hypothesis and its implications for pathogenesis. In *Slow Transmissible Diseases of the Nervous System*, pp. 12–31. Edited by S. B. Prusiner & W. J. Hadlow. New York: Academic Press.

Dickinson, A. G., Fraser, H., Meikle, V. M. & Outram, G. W. (1972). Competition between different scrapie agents in mice. *Nat New Biol* **237**, 244–245.

Dickinson, A. G., Fraser, H., McConnell, I., Outram, G. W., Sales, D. I. & Taylor, D. M. (1975). Extraneural competition between different scrapie agents leading to loss of infectivity. *Nature* **253**, 556.

Garfin, D. E., Stites, D. P., Perlman, J. D., Cochran, S. P. & Prusiner, S. B. (1978). Mitogen stimulation of splenocytes from mice infected with scrapie agent. *J Infect Dis* **138**, 396–400.

Kascsak, R. J., Rubenstein, R., Merz, P. A., Tonna-DeMasi, M., Fersko, R., Carp, R. I., Wisniewski, H. M. & Diringer, H. (1987). Mouse polyclonal and monoclonal antibody to scrapie-associated fibril proteins. *J Virol* **61**, 3688–3693.

Kasper, K. C., Stites, D. P., Bowman, K. A., Panitch, H. & Prusiner, S. B. (1982). Immunological studies of scrapie infection. *J Neuroimmunol* **3**, 187–201.

Kimberlin, R. H. & Walker, C. (1977). Characteristics of a short incubation model of scrapie in the golden hamster. *J Gen Virol* **34**, 295–304.

Kimberlin, R. H. & Walker, C. A. (1985). Competition between strains of scrapie depends on the blocking agent being infectious. *Intervirology* **23**, 74–81.

Kimberlin, R. H. & Walker, C. A. (1986). Pathogenesis of scrapie (strain 263K) in hamsters infected intracerebrally, intraperitoneally or intraocularly. *J Gen Virol* **67**, 255–263.

Kingsbury, D. T., Smeltzer, D. A., Gibbs, C. J., Jr & Gajdusek, D. C. (1981). Evidence for normal cell-mediated immunity in scrapie-infected mice. *Infect Immun* **32**, 1176–1180.

McBride, P. A., Schulz-Schaeffer, W. J., Donaldson, M., Bruce, M., Diringer, H., Kretzschmar, H. A. & Beekes, M. (2001). Early spread of scrapie from the gastrointestinal tract to the central nervous system involves autonomic fibers of the splanchnic and vagus nerves. *J Virol* **75**, 9320–9327.

Manuelidis, L. (1998). Vaccination with an attenuated Creutzfeldt–Jakob disease strain prevents expression of a virulent agent. *Proc Natl Acad Sci U S A* **95**, 2520–2525.

Manuelidis, L. & Yun Lu, Z. (2000). Attenuated Creutzfeldt–Jakob disease agents can hide more virulent infections. *Neurosci Lett* **293**, 163–166.

National Research Council (1996). *Guide for the Care and Use of Laboratory Animals*. Washington, DC: National Academy Press.

Porter, D. D., Porter, H. G. & Cox, N. A. (1973). Failure to demonstrate a humoral immune response to scrapie infection in mice. *J Immunol* **111**, 1407–1410.

Prinz, M., Montrasio, F., Klein, M. A., Schwarz, P., Priller, J., Odermatt, B., Pfeffer, K. & Aguzzi, A. (2002). Lymph nodal prion replication and neuroinvasion in mice devoid of follicular dendritic cells. *Proc Natl Acad Sci U S A* **99**, 919–924.

Prusiner, S. B. (1991). Molecular biology of prion diseases. *Science* **252**, 1515–1522.

Schulz-Schaeffer, W. J., Fatzer, R., Vandevelde, M. & Kretzschmar, H. A. (2000). Detection of PrP(Sc) in subclinical BSE with the paraffin-embedded tissue (PET) blot. *Arch Virol Suppl* **16**, 173–180.

Somerville, R. A., Birkett, C. R., Farquhar, C. F. & 8 other authors (1997). Immunodetection of PrPSc in spleens of some scrapie-infected sheep but not BSE-infected cows. *J Gen Virol* **78**, 2389–2396.

Spongiform Encephalopathy Advisory Council (2001). *Statement on Infectivity in Bovine Tonsil*. London: DEFRA, DH, FSA.

Terry, L. A., Marsh, S., Ryder, S. J., Hawkins, S. A., Wells, G. A. & Spencer, Y. I. (2003). Detection of disease-specific PrP in the distal ileum of cattle exposed orally to the agent of bovine spongiform encephalopathy. *Vet Rec* **152**, 387–392.

Wells, G. A., Hawkins, S. A., Green, R. B., Austin, A. R., Dexter, I., Spencer, Y. I., Chaplin, M. J., Stack, M. J. & Dawson, M. (1998). Preliminary observations on the pathogenesis of experimental bovine spongiform encephalopathy (BSE): an update. *Vet Rec* **142**, 103–106.