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## Prion Interference Is Due to a Reduction in Strain-Specific PrP<sup>Sc</sup> Levels<sup>▽</sup>

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**When two prion strains infect a single host, one strain can interfere with the ability of the other to cause disease but it is not known whether prion replication of the second strain is also diminished. To further investigate strain interference, we infected hamsters in the sciatic nerve with the long-incubation-period transmissible mink encephalopathy (TME) agent DY TME prior to superinfection of hamsters with the short-incubation-period HY TME agent. Increases in the interval between TME agent inoculations resulted in an extension of the incubation period of HY TME or a complete block of the ability of the HY TME agent to cause disease. The sciatic nerve route of inoculation gave the two TME strains access to the same population of neurons, allowing for the potential of prion interference in the lumbar spinal cord. The ability of the DY TME agent to extend the incubation period of HY TME corresponds with detection of DY TME PrP<sup>Sc</sup>, the abnormal isoform of the prion protein, in the lumbar spinal cord. The increased incubation period of HY TME or the inability of the HY TME agent to cause disease in the coinfecting animals corresponds with a reduction in the abundance of HY TME PrP<sup>Sc</sup> in the lumbar spinal cord. When the two strains were not directed to the same populations of neurons within the lumbar spinal cord, interference between HY TME and DY TME did not occur. This suggests that DY TME agent replication interferes with HY TME agent replication when the two strains infect a common population of neurons.**

Prion diseases are a group of emerging, transmissible, neurodegenerative diseases of humans and animals that are inevitably fatal. Two cases of variant Creutzfeldt-Jakob disease (vCJD) were identified in humans who received blood transfusions from asymptomatic individuals who later developed vCJD. These cases suggest that vCJD can be transmitted from human to human via blood prior to the onset of clinical symptoms (27, 32). The recent identification of multiple PrP<sup>Sc</sup> types in vCJD brain tissue suggests that more than one prion strain may be present in field isolates of vCJD (33). Passage of vCJD agents between humans could result in further adaptation of vCJD, resulting in a selection of prion strains that have increased human pathogenicity.

The mechanism of prion adaptation is beginning to be understood. Rodent transmission studies suggest that after intraspecies transmission, prion strains are selected from a mixture or from new strains that arose from a single strain present in the original inoculum (16, 19, 22). Experimental inoculation of individual animals with two prion strains has allowed the biological parameters of strain selection to be characterized. Experimental coinfection of mice with two prion strains was first described with the long-incubation-period scrapie agent strain 22C and the short-incubation-period scrapie agent strain 22A (14). In these experiments, the long-incubation-period strain (i.e., the blocking strain) 22C was intracerebrally inoculated prior to intracerebral inoculation (i.e., superinfection) of the 22A strain. If the two strains acted

independently, the 22A scrapie agent would be expected to cause clinical disease and death of these animals well before the 22C scrapie agent would cause disease. Although the mice succumbed to the 22A strain, based on neuropathological features, the incubation period until the onset of 22A clinical signs was significantly longer than the incubation period for mice inoculated with the 22A scrapie agent alone. Increasing the time interval between the 22C and 22A scrapie agent inoculations resulted in an increase in the incubation period of 22A and even completely inhibited the ability of the 22A scrapie agent to cause disease. This indicated that the blocking strain could interfere with the ability of the superinfecting strain to cause disease, but it is not known whether the blocking strain could interfere with prion replication.

In the present study, we show that the drowsy strain of the transmissible mink encephalopathy (TME) agent (DY TME) can interfere with the hyper strain of the TME agent (HY TME). Infection of the sciatic nerve with the DY TME agent prior to superinfection of hamsters in the sciatic nerve with the HY TME agent can extend the incubation period of the HY TME agent or completely block the ability of the HY TME agent to cause clinical disease. The sciatic nerve route of inoculation directed the two TME strains into the same population of neurons, allowing for the identification of a potential site of prion interference to the lumbar spinal cord. If the two strains were not initially directed to the same populations of neurons, interference between HY TME and DY TME did not occur. The ability of the DY TME agent to extend the incubation period or completely prevent the HY TME agent from causing disease corresponds with a reduction in the accumulation of the HY TME-specific abnormal isoform of the prion protein, PrP<sup>Sc</sup>, in the lumbar spinal cord. These findings sug-

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gest that prion interference is due to a strain-specific reduction in prion replication.

## MATERIALS AND METHODS

**Animal inoculations.** All procedures involving animals complied with the *Guide for the Care and Use of Laboratory Animals* (30) and were approved by the Creighton University institutional animal care and use committee. For these studies, 10- to 11-week-old male Syrian golden hamsters (Harlan Sprague-Dawley) were used. Sciatic nerve inoculations were performed as previously described (5). Briefly, anesthesia was administered by an intraperitoneal injection of a ketamine-xylazine mixture (120 mg of ketamine/kg of body weight, 10 mg of xylazine/kg), and the hamster was restrained on a small-animal retraction system (Fine Science Tools, Foster City, CA). A small incision in the skin of the hind limb was made, and the sciatic nerve was exposed at the popliteal fossa. One microliter containing  $10^{5.2}$  intracerebral (i.c.) 50% lethal doses ( $LD_{50}$ )/g HY TME agent,  $10^{3.1}$  i.c.  $LD_{50}$ /g DY TME agent, or 10% (wt/vol) 10,000-molecular-weight anionic fixable Alexa Fluor 488-conjugated dextran (Molecular Probes, Eugene, OR) was slowly injected with a 30-gauge needle attached to a Hamilton syringe under the perineurium of the sciatic nerve. The 30-gauge needle was reciprocated within the sciatic nerve 10 times to enhance the efficiency of prion infection (5, 17). After inoculation, the wound was closed with surgical staples.

**Clinical diagnosis and calculation of incubation period.** Animals were observed three times per week for the onset of neurological disease, as described previously (8). A clinical diagnosis of HY TME was based on clinical signs of ataxia and hyperexcitability, while clinical diagnosis of DY TME was based on clinical signs of progressive lethargy. The incubation period was defined as the number of days between the inoculation of the agent that resulted in clinical signs and the onset of clinical signs.

**Tissue collection.** Hamsters were killed at selected time points or during the clinical course of disease by  $CO_2$  asphyxiation in an animal holding chamber, as recommended by the American Veterinary Medical Association Panel on Euthanasia. Brains, brainstems, and spinal cords were collected as described previously (5). The L4 to L6 lumbar spinal cord segments, containing the ventral motor neuron (VMN) cell bodies whose axons project into the sciatic nerve, were collected as previously described (5). The intervertebral disk between T6 and T7 and the intervertebral disk between T10 and T11 serve as the rostral and caudal landmarks for lumbar spinal cord collection, respectively (5). The tissue was either flash frozen for Western blot analysis or perfused for microscopy.

**Tissue preparation and Western blot analysis.** Brain and spinal cord material was homogenized to 10% (wt/vol) by passage of the tissue through a 26-gauge needle in Dulbecco's phosphate-buffered saline (DPBS) without  $Ca^{++}$  or  $Mg^{++}$  (Mediatech, Herndon, VA), followed by a 30-second incubation in a cup horn sonicator (Fisher Scientific, Atlanta, GA). The tissue was diluted to 5% (wt/vol) in DPBS containing 4 or 40 units of proteinase K (PK) (Roche Diagnostics Corporation, Indianapolis, IN) and incubated at 37°C for 1 h with constant agitation. The PK digestion was terminated by the addition of Pefablock SC (Roche Diagnostics Corporation, Indianapolis, IN) to a final concentration of 1 mM. This digestion condition resulted in the detection of only the PK-resistant population of PrP<sup>Sc</sup> and in the digestion of the normal form of the prion protein, PrP<sup>C</sup> (11). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis were performed as described previously (4) with the monoclonal anti-PrP antibody 3F4 (Chemicon, Temecula, CA) or with the R30 antibody, which is a polyclonal serum directed against bovine PrP sequence 89 to 103 (12). To more clearly illustrate the differences in molecular weight or the antibody binding properties of PrP<sup>Sc</sup>, multiple animals are shown in each group. The Western blot was developed with Pierce Supersignal West Femto maximum-sensitivity substrate, according to the manufacturer's instructions (Pierce, Rockford, IL), and imaged on a Kodak 2000R imaging station (Kodak, Rochester, NY). The quantification of PrP<sup>Sc</sup> abundance and the comparison of PrP<sup>Sc</sup> abundance between samples was performed as described previously (5).

**PrP<sup>Sc</sup> immunohistochemistry.** Immunodetection of PrP<sup>Sc</sup> in the central nervous system (CNS) was performed as previously described (4). Briefly, hamsters were perfused intracardially with DPBS prior to perfusion with McLean's paraformaldehyde-lysine-periodate (PLP). The tissues were removed from the animal and immersion fixed in PLP for 5 to 7 h at room temperature, placed in 70% (vol/vol) ethyl alcohol, and embedded in paraffin (36). Following rehydration of the tissue sections (7  $\mu$ m), antigen retrieval was performed by incubation of the sections in 95% formic acid (Sigma-Aldrich, St. Louis, MO) for 20 min at room temperature. PrP<sup>Sc</sup> was detected with the monoclonal anti-PrP antibody 3F4 (Chemicon, Temecula, CA) and visualized by the ABC-horseradish peroxidase Elite staining method (Vector Laboratories, Burlingame, CA). The chromogen was developed with 0.05% (wt/vol) diaminobenzidine (Sigma-Aldrich, St. Louis,

MO) in Tris-buffered saline containing 0.003%  $H_2O_2$  and counter stained with hematoxylin (Richard Allen Scientific, Kalamazoo, MI). A minimum of three infected and two uninfected animals per group and a minimum of 80 tissue sections of lumbar spinal cord per animal were examined. Microscopy was performed with a Nikon i80 microscope (Nikon, Melville, NY), and images were captured with a DigiFire camera and ImageSys digital imaging software (Soft Imaging Systems GmbH).

**Fluorescence microscopy of retrograde tracer.** Hamsters ( $n = 5$ ) were injected in the sciatic nerve with 1  $\mu$ l of a 10% (wt/vol) solution of 10,000-molecular-weight lysine-fixable dextran conjugated to Alexa Fluor 488, sacrificed 2 weeks postinjection, and perfused with DPBS followed by PLP fixative, as previously described (4). The lumbar spinal cord was removed and immersed in 20% (wt/vol) sucrose for 24 h prior to serial sectioning (60  $\mu$ m) of the entire lumbar spinal cord (vertebral level, T10 to T13) on a freezing microtome. Microscopy and imaging were performed as described above.

**Statistical analysis.** Linear regression and Mann-Whitney rank sum analyses were performed with Prism 4.0 for Macintosh software (GraphPad Software, Inc., San Diego, CA). Incubation period data and PrP<sup>Sc</sup> abundance data from hamsters infected with the HY TME and DY TME agents were compared with a two-tailed Mann-Whitney rank sum test. A  $P$  value of  $\leq 0.01$  was used in all comparisons to determine whether the two datasets were statistically different.

## RESULTS

### Prion strain interference following sciatic nerve inoculation.

We had previously shown that sciatic nerve inoculation of the DY TME agent 30 or 60 days prior to sciatic nerve superinfection of hamsters with the HY TME agent did not extend the incubation period of HY TME (2). Expanding on these previous studies, the DY TME agent was inoculated into the sciatic nerve and, at 60, 90, or 120 days after DY TME agent infection, the hamsters were superinfected in the same sciatic nerve with the HY TME agent. If no interaction between the DY TME and HY TME agents occurred, then the HY TME agent would be expected to cause clinical disease prior to the onset of DY TME clinical signs. As outlined in Table 1, all five hamsters inoculated with the DY TME agent 60 days prior to superinfection of hamsters with the HY TME agent developed clinical signs of HY TME with an incubation period of  $78 \pm 3$  days, which is not different ( $P > 0.05$ ) from the results for control hamsters inoculated in the sciatic nerve with the HY TME agent alone. Sciatic nerve inoculation of the DY TME agent 90 days prior to sciatic nerve superinfection of hamsters with the HY TME agent resulted in all six hamsters developing clinical signs of HY TME with an incubation period of  $90 \pm 7$  days, which is 12 days longer than for control hamsters inoculated with the HY TME agent alone (Table 1) ( $P < 0.01$ ). Finally, sciatic nerve inoculation of the DY TME agent 120 days prior to sciatic nerve superinfection with the HY TME agent resulted in all five hamsters developing clinical signs of DY TME with an incubation period of  $220 \pm 3$  days, which was not different ( $P > 0.05$ ) from the results for control hamsters inoculated with the DY TME agent alone (Table 1). In the 120-day-interval group, the time between superinfection of hamsters with the HY TME agent and the onset of DY TME clinical signs was 100 days. This is 22 days past the incubation period for hamsters inoculated with the HY TME agent alone (Table 1). Western blot analysis of PK-digested brain homogenate indicated that animals with HY TME clinical signs had corresponding HY TME-specific PrP<sup>Sc</sup> (fastest-migrating polypeptide, 21 kDa [Fig. 1, lanes 5 to 11]), and animals with DY TME clinical signs had DY TME-specific PrP<sup>Sc</sup> (fastest-migrating polypeptide, 19 kDa [Fig. 1, lanes 3 to 4 and 12 to 13]), confirming the clinical diagnosis (Fig. 1). The abundance

TABLE 1. Clinical signs of and incubation periods for hamsters inoculated in the sciatic nerve with the DY TME agent prior to superinfection of hamsters with the HY TME agent

| First inoculation | Interval between inoculations (days) | Second inoculation | Clinical sign | PrP <sup>Sc</sup> migration (kDa) | No. of hamsters affected/no. inoculated | Onset of clinical symptoms (avg days postinfection ± SD) <sup>a</sup> |                          |
|-------------------|--------------------------------------|--------------------|---------------|-----------------------------------|---|---|--------------------------|
|                   |                                      |                    |               |                                   |   | After first inoculation   | After second inoculation |
| DY TME agent      | 120                                  | Mock               | DY TME        | 19                                | 5/5                                     | 217 ± 2   | NA                       |
| Mock              | 120                                  | HY TME agent       | HY TME        | 21                                | 5/5                                     | NA  | 78 ± 2                   |
| DY TME agent      | 60                                   | HY TME agent       | HY TME        | 21                                | 5/5                                     | 138 ± 3   | 78 ± 3*                  |
| DY TME agent      | 90                                   | HY TME agent       | HY TME        | 21                                | 6/6                                     | 180 ± 7   | 90 ± 7†                  |
| DY TME agent      | 120                                  | HY TME agent       | DY TME        | 19                                | 5/5                                     | 220 ± 3‡  | 100 ± 3                  |

<sup>a</sup> NA, not applicable; \*, incubation period similar to that for animals inoculated with the HY TME agent alone (*P* > 0.05); †, incubation period longer than that for animals inoculated with the HY TME agent alone (*P* < 0.01); ‡, incubation period similar to that for animals inoculated with the DY TME agent alone (*P* > 0.05).

of PrP<sup>Sc</sup> from the brains of coinfectd hamsters was similar to the levels in control hamsters (Fig. 1).

**Western blot detection of DY TME PrP<sup>Sc</sup> in the spinal cord.** The initial detection and spread of DY TME PrP<sup>Sc</sup> in the spinal cord after sciatic nerve inoculation was determined by PrP<sup>Sc</sup> Western blot analysis. Western blot analysis of PK-digested spinal cord homogenate failed to detect PrP<sup>Sc</sup> at 60 days after DY TME agent infection, consistent with our previous studies (2) (Fig. 2A). At 90 days after DY TME agent infection, PrP<sup>Sc</sup> was detected in vertebral segments C5 to C7 through T10 to T13. The highest levels of PrP<sup>Sc</sup> were in the T10 to T13 vertebral segment, a result consistent with the DY TME agent entering the CNS in the lumbar spinal cord following sciatic nerve inoculation (Fig. 2B). At 120 days after DY TME agent infection, PrP<sup>Sc</sup> was detected in the entire length of the spinal cord (Fig. 2C). In each vertebral segment examined, there was an increase in the PrP<sup>Sc</sup> abundance in hamsters inoculated with the DY TME agent at 120 days postinfection compared to the level at 90 days postinfection (Fig. 2B and C).

**Differential detection of DY TME and HY TME PrP<sup>Sc</sup> by Western blot analysis.** Western blot analysis of PK-digested (4 U/ml) HY TME- or DY TME-infected brain homogenates with the anti-PrP monoclonal antibody 3F4 resulted in detec-

tion of HY TME and DY TME PrP<sup>Sc</sup> (Fig. 3, lanes 1 and 2). The levels of DY TME and HY TME PrP<sup>Sc</sup> were similar, but DY TME PrP<sup>Sc</sup> migrated 1 to 2 kDa faster than HY TME PrP<sup>Sc</sup> (Fig. 3, lanes 1 and 2) (6). This difference in migration is due to differences in the PK cleavage sites of HY TME and DY TME PrP<sup>Sc</sup> (7). To determine the abundance of HY TME and DY TME PrP<sup>Sc</sup> in the coinfectd animals, we expanded on

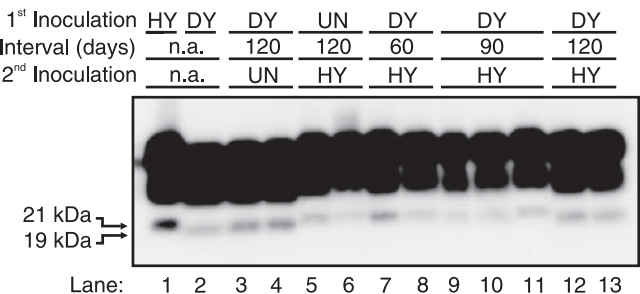


FIG. 1. HY TME and DY TME strain-specific PrP<sup>Sc</sup> properties correspond with the clinical signs of disease in hamsters infected with the DY TME agent at 60, 90, or 120 days prior to superinfection of hamsters with the HY TME agent. In control animals inoculated with only the HY TME agent (lanes 1, 5, and 6), the nonglycosylated PrP<sup>Sc</sup> polypeptide migrates at 21 kDa, compared to the 19-kDa migration of PrP<sup>Sc</sup> from control animals inoculated only with the DY TME agent (lanes 2, 3, and 4). Migration of the 19- and 21-kDa nonglycosylated PrP<sup>Sc</sup> polypeptides is indicated to the left of the figure. UN, mock inoculation; HY, HY TME agent; DY, DY TME agent.

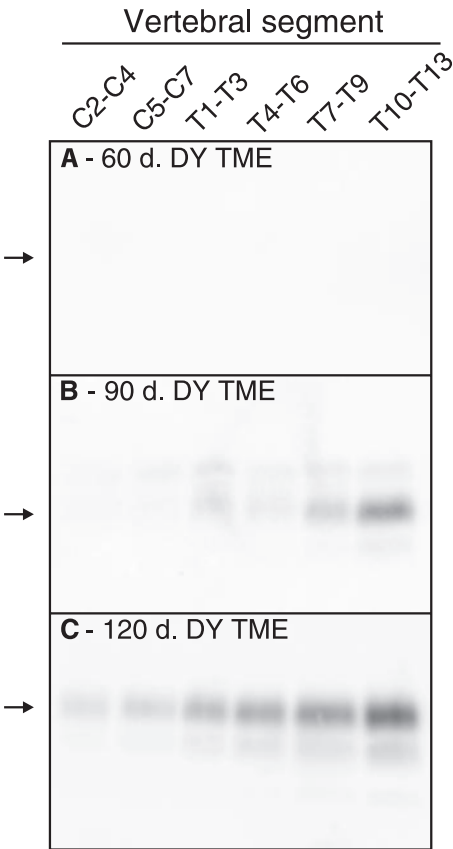


FIG. 2. Abundance of PrP<sup>Sc</sup> in the spinal cord following sciatic nerve inoculation with the DY TME agent. Shown is PrP<sup>Sc</sup> Western blot analysis of spinal cord homogenates digested with PK at 60 (A), 90 (B), and 120 (C) days after DY TME infection of the sciatic nerve. Cervical (C) and thoracic (T) vertebral spinal cord segments are indicated at the top of the figure. The arrows at the left of the figure indicate migration of the 29-kDa molecular mass marker.



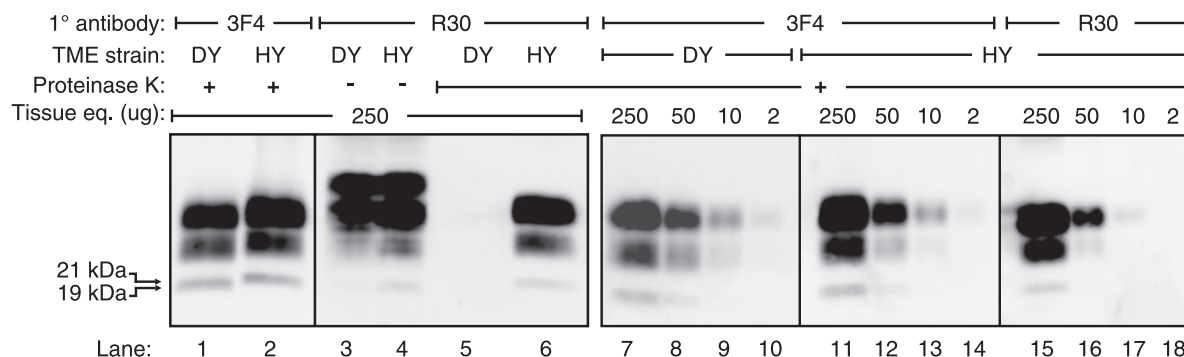


FIG. 3. Detection and differentiation of HY TME and DY TME PrP<sup>Sc</sup> by Western blot analysis. PK digestion of HY TME and DY TME PrP<sup>Sc</sup> results in DY TME PrP<sup>Sc</sup> migrating 1 to 2 kDa faster than HY TME PrP<sup>Sc</sup> with the 3F4 antibody (lanes 1 and 2). The R30 antibody epitope is present on both HY TME and DY TME PrP<sup>Sc</sup> prior to PK digestion (lanes 3 and 4). PK digestion removes the R30 epitope from DY TME PrP<sup>Sc</sup> (lane 5) but not from HY TME PrP<sup>Sc</sup> (lane 6). The 3F4 antibody can detect similar levels of HY TME and DY TME PrP<sup>Sc</sup> (lanes 7 to 14). The R30 antibody can detect levels of HY TME PrP<sup>Sc</sup> similar to those detected by the anti-PrP antibody 3F4 (lanes 11 to 18). Migration of the 19- and 21-kDa nonglycosylated PrP<sup>Sc</sup> polypeptides is indicated to the left of the figure.

previous studies describing differences in antibody binding to the HY TME and DY TME PrP<sup>Sc</sup> polypeptides (6). To differentiate HY TME PrP<sup>Sc</sup> from DY TME PrP<sup>Sc</sup> based on antibody binding, we used the R30 polyclonal serum. Western blot analysis of 250- $\mu$ g equivalents of non-PK-digested HY TME or DY TME agent-infected brain homogenate with R30 detected PrP<sup>Sc</sup> from each TME agent strain and PrP<sup>C</sup> (Fig. 3, lanes 3 and 4). Western blot analysis of HY TME or DY TME agent-infected homogenates digested with PK (40 U/ml) using the R30 antibody resulted in HY TME PrP<sup>Sc</sup> detection (Fig. 3, lane 6), but we were unable to detect DY TME PrP<sup>Sc</sup> with the R30 antibody (Fig. 3, lane 5), presumably due to the digestion of the R30 epitope on the DY TME PrP<sup>Sc</sup> molecule. Western blot analysis of fivefold serial dilutions of DY TME (Fig. 3, lanes 7 to 10) and HY TME (Fig. 3, lanes 11 to 14) agent-infected brain homogenate with the 3F4 antibody resulted in the detection of PrP<sup>Sc</sup> in 2- $\mu$ g equivalents of both HY TME and DY TME brain homogenates. Western blot analysis of HY TME agent-infected brain homogenate with the R30 antibody (Fig. 3, lanes 15 to 18) detected HY PrP<sup>Sc</sup> in 2- $\mu$ g brain equivalents, indicating that R30 has an ability to detect HY PrP<sup>Sc</sup> similar to that of 3F4. The presence of DY PrP<sup>Sc</sup> did not interfere with the ability of R30 to detect HY PrP<sup>Sc</sup> (data not shown).

**PrP<sup>Sc</sup> Western blot analysis of lumbar spinal cords from hamsters inoculated in the sciatic nerve with the DY TME and HY TME agents.** To investigate the effects of DY TME agent infection on the initial detection and rate of accumulation of HY TME PrP<sup>Sc</sup> in the lumbar spinal cord, hamsters were inoculated in the right sciatic nerve with the DY TME agent (experimental group) or with the same concentration of an uninfected homogenate (control group) 90 days prior to superinfection of hamsters in the right sciatic nerve with the HY TME agent. At 4, 6, 8, and 10 weeks after HY TME agent infection, three hamsters were sacrificed in the control group and three animals were sacrificed in the experimental group. Western blot analysis with the R30 antibody was performed on 500- $\mu$ g equivalents of PK-digested lumbar cord homogenate. In both the control and experimental groups, HY TME PrP<sup>Sc</sup> was first detected at 6 weeks postinfection and the PrP<sup>Sc</sup> abun-

dance increased through 10 weeks after HY TME superinfection (Fig. 4). At both 8 and 10 weeks after sciatic nerve superinfection with the HY TME agent, the abundance of HY TME PrP<sup>Sc</sup> in the lumbar spinal cord from the control group was greater than in the experimental group (Fig. 4). Linear regression analysis of the control group ( $r^2 = 0.9997$ ) from weeks 6 to 10 after HY TME agent superinfection indicated a zero intercept of 5 weeks and 6 days and an HY TME PrP<sup>Sc</sup> doubling time of 4.3 days. In the experimental group, linear regression analysis ( $r^2 = 0.8941$ ) from weeks 6 to 10 after HY TME superinfection indicated a zero intercept of 6 weeks and 2 days and an HY TME PrP<sup>Sc</sup> doubling time of 6.5 days. The regression analysis indicated that sciatic nerve infection with the DY TME agent at 90 days prior to sciatic nerve superinfection with the HY TME agent delayed the initial detection of HY PrP<sup>Sc</sup> in the lumbar spinal cord by 3 days and resulted in a 51% reduction in the rate of HY PrP<sup>Sc</sup> accumulation.

To determine whether the HY TME agent could establish infection in the lumbar spinal cord in hamsters inoculated with the DY TME agent 120 days prior to superinfection of hamsters with the HY TME agent, Western blot analysis of 250- $\mu$ g equivalents of lumbar spinal cord with the 3F4 and R30 antibodies was performed on animals at the terminal stage of disease. In hamsters inoculated with the DY TME agent 120 days prior to sciatic nerve superinfection with the HY TME agent, DY TME-specific PrP<sup>Sc</sup> was detected in the lumbar spinal cord with the 3F4 antibody (Fig. 3 and Fig. 5, lanes 5 and 6), suggesting that HY TME PrP<sup>Sc</sup> levels were reduced. Western blot analysis of the same tissues was performed with the R30 antibody, and HY TME PrP<sup>Sc</sup> was not detected (Fig. 5, lanes 11 and 12). To confirm the failure to detect HY TME PrP<sup>Sc</sup> in the lumbar spinal cord from the 120-day coinfecting group, this experiment was repeated in its entirety, as described above. In the control group inoculated with the HY TME agent alone, we were able to detect HY PrP<sup>Sc</sup> with the 3F4 antibody (Fig. 5, lanes 13 and 14). In the experimental group, we were unable to detect HY PrP<sup>Sc</sup> in 250- $\mu$ g equivalents of PK-digested lumbar spinal cord homogenate with the R30 antibody (Fig. 5, lanes 17 and 18) but high levels of DY

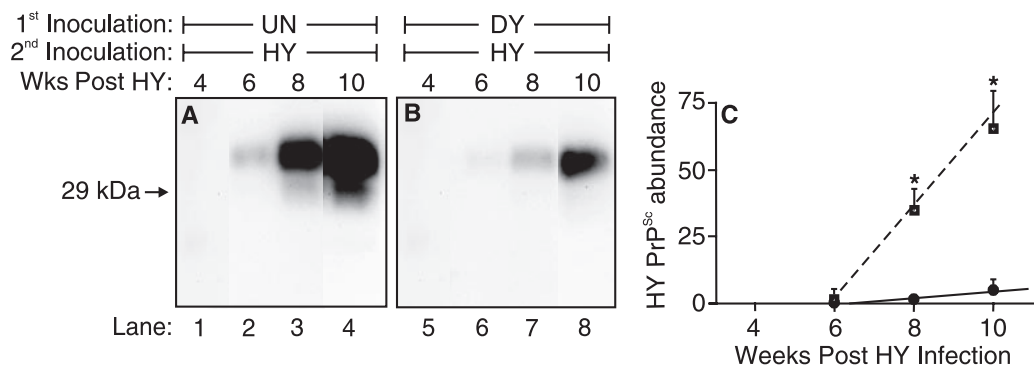


FIG. 4. Sciatic nerve inoculation with the DY TME agent 90 days prior to sciatic nerve superinfection of hamsters with the HY TME agent results in a reduction in HY TME PrP<sup>Sc</sup> in the lumbar spinal cord. Shown are immunodetection of HY TME PrP<sup>Sc</sup> in the lumbar spinal cord by Western blotting with the R30 antibody (A and B) and quantification of HY TME PrP<sup>Sc</sup> polypeptides at selected time points after HY TME agent inoculation (C). (A) Control hamsters were inoculated in the sciatic nerve with an uninfected brain homogenate 90 days prior to sciatic nerve inoculation with the HY TME agent. (B) In the experimental group, hamsters were inoculated with the DY TME agent 90 days prior to sciatic nerve superinfection of hamsters with the HY TME agent. The arrow on left of the Western blot indicates the migration of the 29-kDa molecular mass marker. UN, mock infection; HY, HY TME agent; DY, DY TME agent. (C) In control hamsters (squares), HY TME PrP<sup>Sc</sup> levels were significantly higher ( $P < 0.01$ ) at 8 and 10 weeks postinfection, as indicated by asterisks, than were the HY TME PrP<sup>Sc</sup> levels in the experimental group (circles). Bars at each time point represent standard deviations. Linear regression analyses of HY TME PrP<sup>Sc</sup> abundance between 6 and 10 weeks after HY TME agent infection in the control group (dashed line) and in the experimental group (solid line) are indicated.

TME PrP<sup>Sc</sup> were identified with the 3F4 antibody (Fig. 5, lanes 15 and 16).

Based on the abundance of HY TME PrP<sup>Sc</sup> in the lumbar spinal cord from hamsters inoculated with the HY TME agent alone (Fig. 5, lanes 7 to 8 and 13 to 14), there is a minimum of a 100-fold reduction in HY TME PrP<sup>Sc</sup> levels in hamsters that were inoculated with the DY TME agent 120 days prior to superinfection of hamsters with the HY TME agent (Fig. 3, lanes 15 to 18, and Fig. 5, lanes 11 to 12 and 17 to 18). Western blot analysis of the same tissue with the 3F4 antibody revealed DY TME-specific PrP<sup>Sc</sup> (Fig. 5, lanes 5, 6, 15, and 16). Due to the presence of DY TME PrP<sup>Sc</sup> in these tissues, an animal bioassay would have a reduced ability to detect small amounts of the HY TME agent (3). Therefore, it is not possible to unequivocally demonstrate whether the HY TME agent is present in the lumbar spinal cord.

**Immunohistochemical detection of PrP<sup>Sc</sup> in the lumbar spinal cord.** To further refine the locations of TME interfer-

ence, PrP<sup>Sc</sup> immunohistochemistry was performed on lumbar spinal cord from hamsters inoculated in the sciatic nerve with either the DY TME or HY TME agent. In animals inoculated in the sciatic nerve with the HY TME agent, HY PrP<sup>Sc</sup> was first detected at 2 weeks postinfection in the lumbar spinal cord ipsilateral to the site of inoculation within the soma of VMNs and in the surrounding neuropil (Fig. 6B and C). The location and morphology of the cell types that contain HY PrP<sup>Sc</sup> in the lumbar cord at 2 weeks after HY TME agent infection were similar to fluorescently labeled VMNs (Fig. 6A).

Following sciatic nerve inoculation of the DY TME agent, PrP<sup>Sc</sup> was not detected at 60 days postinfection in the lumbar spinal cord, consistent with Western blot data (2) (Fig. 2A). Using PrP<sup>Sc</sup> immunohistochemistry, DY TME PrP<sup>Sc</sup> was detected at 90 days postinfection in the lumbar spinal cord, consistent with Western blot data (Fig. 2B). Within the lumbar spinal cord, DY TME PrP<sup>Sc</sup> was detected in lamina IX, which contains VMNs, and in the medial regions of laminae II, III,

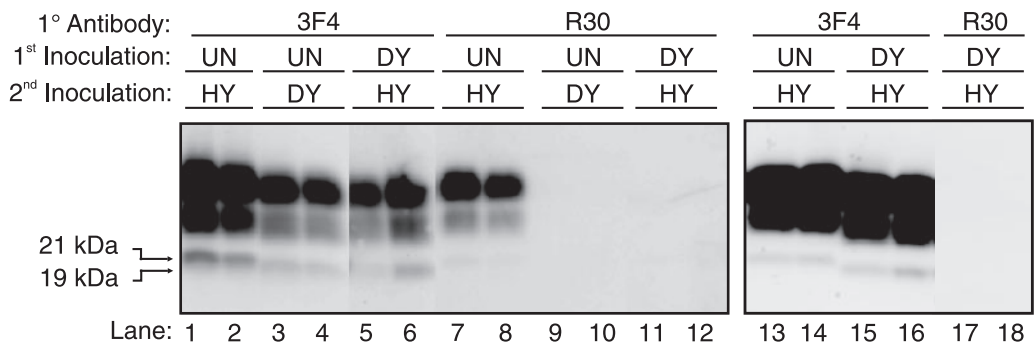


FIG. 5. Sciatic nerve inoculation with the DY TME agent 120 days prior to sciatic nerve superinfection of hamsters with the HY TME agent results in a failure to detect HY TME PrP<sup>Sc</sup> in the lumbar spinal cord at terminal disease. Shown is PrP<sup>Sc</sup> Western blot analysis of lumbar spinal cord homogenates (250-μg equivalents) digested with PK from hamsters infected with the HY TME agent or the DY TME agent or from animals inoculated with the DY TME agent 120 days prior to sciatic nerve superinfection of hamsters with the HY TME agent using the 3F4 or R30 antibody. Migration of the 19- and 21-kDa nonglycosylated PrP<sup>Sc</sup> polypeptides is indicated to the left of the figure. UN, mock infection; HY, HY TME agent; DY, DY TME agent. Lanes 1 to 12, experiment 1; lanes 13 to 18, experiment 2.

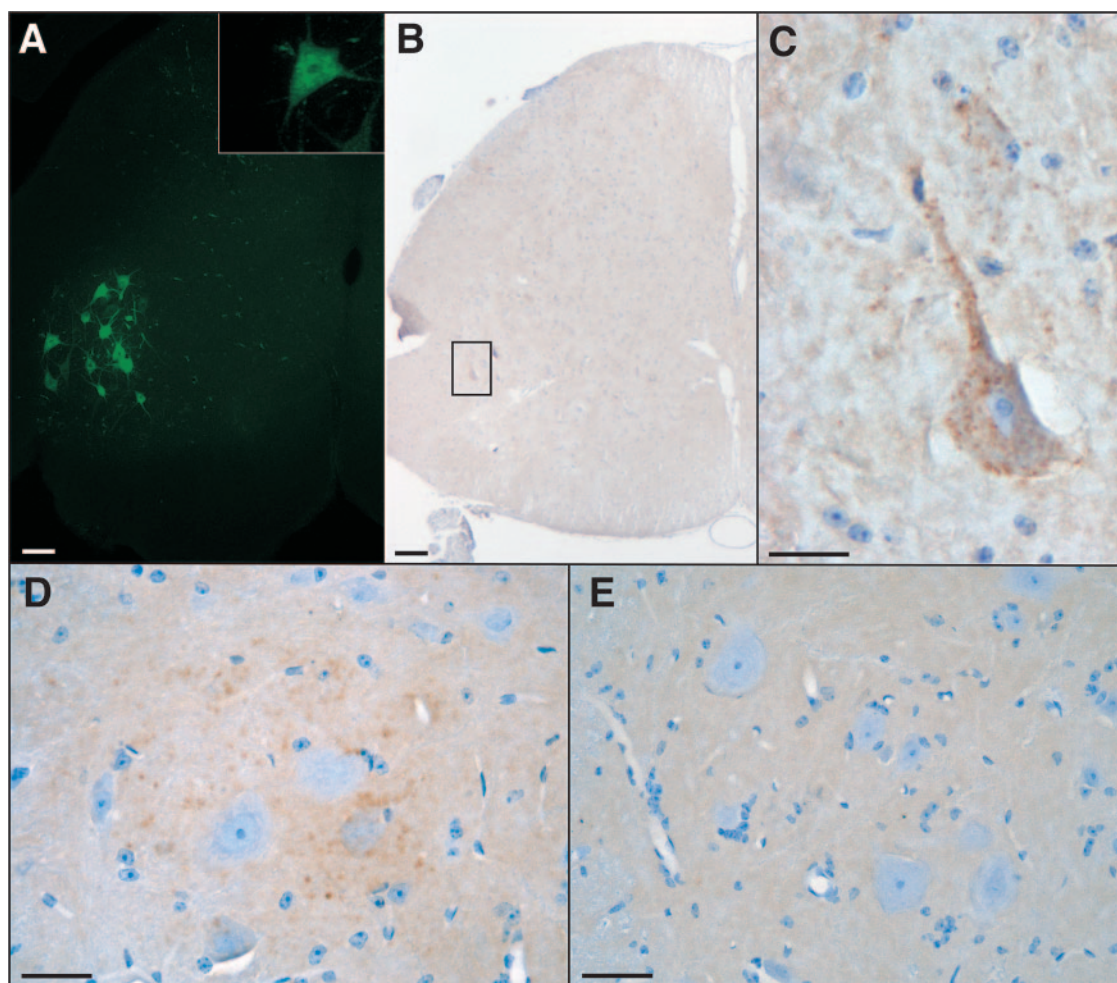


FIG. 6. Initial deposition of HY TME and DY TME PrP<sup>Sc</sup> following sciatic nerve inoculation is in the VMNs of the lumbar spinal cord. (A) Fluorescence microscopy of lumbar spinal cord ipsilateral to sciatic nerve injection of fluorescently tagged dextran. Dextran is specifically transported to VMN cell bodies in spinal cord lamina IX. The insert depicts a single VMN cell body that is filled with the retrograde tracer. (B) Low-power image of PrP<sup>Sc</sup> immunohistochemistry from the lumbar spinal cord ipsilateral to the site of HY TME agent inoculation at 2 weeks postinfection. (C) PrP<sup>Sc</sup> immunohistochemistry of the boxed region in panel B of a VMN with extensive intrasomal HY TME PrP<sup>Sc</sup> deposits in lamina IX. (D) PrP<sup>Sc</sup> immunohistochemistry of lumbar spinal cord lamina IX ipsilateral to the site of DY TME agent inoculation at 90 days postinfection, indicating DY TME PrP<sup>Sc</sup> deposition associated with VMNs. (E) PrP<sup>Sc</sup> immunohistochemistry of lumbar spinal cord lamina IX ipsilateral to mock inoculation of uninfected brain homogenate. Scale bars: panels A and B, 100  $\mu$ m; panel C, 25  $\mu$ m; panels D and E, 50  $\mu$ m.

IV, and X in the lumbar spinal cord ipsilateral to the site of inoculation, where it was not detected in mock-infected hamsters (Fig. 6D and E and data not shown). At 120 days after DY TME agent infection, PrP<sup>Sc</sup> had spread to lamina V, ipsilateral to the site of DY TME agent inoculation (data not shown). The initial detection and spread of PrP<sup>Sc</sup> in the lumbar spinal cord was consistent with detection of PrP<sup>Sc</sup> by Western blotting (Fig. 2). These data are consistent with entry of the HY TME and DY TME agents into the CNS via VMNs and suggest a potential location for TME agent interference to occur.

**Prion strain interference in hamsters coinfecting with the DY TME agent in the right sciatic nerve and with the HY TME agent in the left sciatic nerve.** If DY TME agent replication, ipsilateral to the site of inoculation, is responsible for diminishing the ability of the HY TME agent to cause disease, then inoculation of the HY TME agent in the sciatic nerve con-

tralateral to DY TME agent inoculation should result in animals succumbing to HY TME with incubation periods similar to those for animals inoculated with the HY TME agent alone. To test this hypothesis, hamsters were either mock or DY TME agent inoculated in the right sciatic nerve 90 or 120 days prior to superinfection of hamsters with the HY TME agent in the left (contralateral) sciatic nerve.

Hamsters inoculated with the DY TME agent in the right sciatic nerve 90 days prior to left sciatic nerve (contralateral) superinfection with the HY TME agent developed HY TME clinical signs, and HY TME PrP<sup>Sc</sup> was detected in the brain by Western blotting (Table 2 and data not shown). The incubation period for these animals was  $77 \pm 3$  days and was not significantly different from that for control hamsters inoculated with the HY TME agent alone ( $77 \pm 3$  days;  $P > 0.05$ ). For hamsters inoculated with the DY TME agent in the right sciatic nerve 120 days prior to left sciatic nerve (contralateral) super-



TABLE 2. Clinical signs of and incubation periods for hamsters inoculated in the right sciatic nerve with the DY TME agent prior to superinfection in the left sciatic nerve with the HY TME agent

| First inoculation<br>(right sciatic nerve) | Interval between<br>inoculations<br>(days) | Second inoculation<br>(left sciatic nerve) | Clinical sign | PrP <sup>Sc</sup><br>migration<br>(kDa) | No. of hamsters<br>affected/no.<br>inoculated | Onset of clinical symptoms<br>(avg days postinfection $\pm$ SD) <sup>a</sup> |                             |
|--|--|--|---------------|---|---|--|-----------------------------|
|  |  |  |               |   |   | After first<br>inoculation   | After second<br>inoculation |
| Mock                                       | 90   | HY TME agent                               | HY TME        | 21                                      | 5/5   | NA   | 77 $\pm$ 3                  |
| DY TME agent                               | 90   | HY TME agent                               | HY TME        | 21                                      | 5/5   | 167 $\pm$ 3  | 77 $\pm$ 3*                 |
| DY TME agent                               | 90   | Mock                                       | DY TME        | 19                                      | 5/5   | 229 $\pm$ 3  | NA                          |
| Mock                                       | 120  | HY TME agent                               | HY TME        | 21                                      | 5/5   | NA   | 73 $\pm$ 3                  |
| DY TME agent                               | 120  | HY TME agent                               | HY TME        | 21                                      | 5/5   | 197 $\pm$ 11   | 77 $\pm$ 11                 |
| DY TME agent                               | 120  | Mock                                       | DY TME        | 19                                      | 4/4   | 232 $\pm$ 4  | NA                          |

<sup>a</sup> NA, not applicable; \*, incubation period similar to that for animals inoculated with the HY TME agent alone ( $P > 0.05$ ).

infection with the HY TME agent, all five animals developed HY TME clinical signs and HY PrP<sup>Sc</sup> was detected in the brain by Western blotting (Table 2 and data not shown). The incubation period for these animals was 77  $\pm$  11 days and was not significantly different from that for control hamsters inoculated with the HY TME agent alone (77  $\pm$  3 days;  $P > 0.05$ ). These data are consistent with TME agent strain selection occurring in the ipsilateral lumbar spinal cord.

## DISCUSSION

DY TME agent infection of the sciatic nerve can either extend the incubation period for hamsters superinfected with the HY TME agent or completely block the ability of the HY TME agent to cause clinical disease. The interval between DY TME and HY TME agent inoculations determines the clinical outcome of disease. This effect has been observed in mice and hamsters intracerebrally or intraperitoneally coinfecting with strains of scrapie, TME, and CJD (2, 3, 13, 14, 28). This study selectively measured HY TME and DY TME PrP<sup>Sc</sup> in the CNS of coinfecting animals based on their unique biochemical properties. In animals infected with the DY TME and HY TME agents, detection of DY TME PrP<sup>Sc</sup> corresponds with a reduction in HY TME PrP<sup>Sc</sup> abundance and a corresponding increase in the HY TME incubation period or a complete failure of HY TME to cause disease. Since there is compelling evidence to suggest that the abnormal isoform of the prion protein is the infectious agent in prion diseases, our data indicate that the DY TME agent can interfere with HY TME agent replication (10, 26, 34).

The ability of the DY TME agent to extend the incubation period for hamsters superinfected with the HY TME agent corresponded with the detection of DY TME PrP<sup>Sc</sup> in the lumbar spinal cord. This finding extends the results of previous studies by demonstrating that the prion strain that was inoculated first (i.e., the blocking strain) must be able to replicate to have an effect on the subsequently inoculated prion strain (2, 18, 35). The initial detection of DY TME PrP<sup>Sc</sup> and the increase in abundance of DY TME PrP<sup>Sc</sup> corresponded with the ability of the DY TME agent to interfere with the HY TME agent. The evidence in this and other animal models of prion disease is in contrast to a cell culture model of prion strain coinfection, wherein detection of PrP<sup>Sc</sup> does not correspond with the ability of the blocking strain to affect the superinfect-

ing strain (31). While the reason for this discrepancy is not known, it could be due to host and strain differences between these model systems.

DY TME agent replication can reduce, or possibly block, HY TME agent replication in the CNS. A 90-day interval between DY TME agent infection and superinfection of hamsters with the HY TME agent resulted in animals succumbing to HY TME agent infection with an incubation period greater than animals inoculated with the HY TME agent alone. In this group of coinfecting animals, HY PrP<sup>Sc</sup> is first detected in the lumbar spinal cord at the same time point after HY TME agent infection as animals infected with the HY TME agent alone. These data suggest that prior infection with the DY TME agent does not affect the transport of the HY TME agent from the site of inoculation in the sciatic nerve to VMNs in the lumbar spinal cord, indicating that the sciatic nerve would not be a location of prion strain interference. The reduced rate of HY PrP<sup>Sc</sup> accumulation in the lumbar spinal cord of hamsters first inoculated with the DY TME agent suggests that DY TME agent replication interferes with HY TME agent replication. Similarly, the inability to detect HY PrP<sup>Sc</sup> in the lumbar spinal cord in the 120-day-interval coinfecting group suggests that DY TME agent replication blocks HY TME agent replication. Since Western blot analysis measures PrP<sup>Sc</sup> abundance only, it is possible that prior infection with the DY TME agent increases the clearance of HY PrP<sup>Sc</sup> or prevents its spread to second-order neurons within the lumbar spinal cord. Irrespective of the precise mechanism of interference involved, the reduction in HY TME PrP<sup>Sc</sup> abundance predicts the observed increase in incubation period of HY TME in the 90-day-interval group and the ability of the DY TME agent to cause disease in the 120-day-interval group.

The results of this study indicate that prion interference occurs in neurons located in the CNS. Prion interference occurred in the lumbar cord ipsilateral to the site of inoculation, based on data from Western blot analyses of the lumbar spinal cords of coinfecting animals and from coinfection experiments in which both the right and left sciatic nerves were inoculated. Further narrowing of the location of prion interference is based on the known neuroanatomical pathways that the HY TME and DY TME agents utilize following sciatic nerve inoculation. The HY TME agent is transported within the CNS via three descending motor tracts (5). These motor tracts either directly synapse on VMNs or synapse on interneurons that

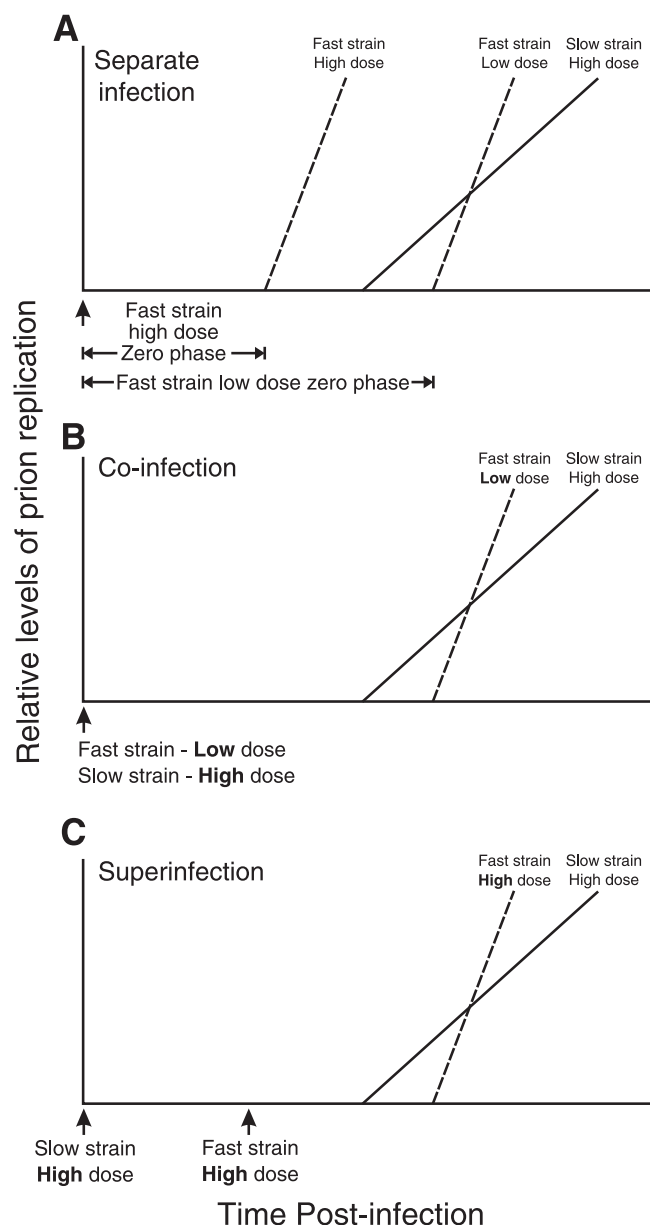


FIG. 7. The relative times of onset of prion replication can be similar in coinfecting and superinfected hosts. (A) The difference in incubation period between a high and low dose of a single prion strain is due to an extension of the zero phase. Once prion replication is detected, the kinetics of prion replication between high and low doses are similar. Prion strains can also exhibit differences in the duration of the zero phase and rate of prion replication and/or PrP<sup>Sc</sup> accumulation. Infection of two strains at the same time (coinfection) (B) or infection with one strain followed by infection with a second strain (superinfection) (C) can result in the same relative onset of replication between the two strains. The dose of the agent (B) or the interval between infections with the two strains (C) influences the onset of prion replication.

in turn synapse on VMNs of the lumbar spinal cord (1, 9, 23, 24). Consistent with this finding, HY TME PrP<sup>Sc</sup> is first detected in VMNs of the lumbar spinal cord ipsilateral to the site of inoculation. DY TME PrP<sup>Sc</sup> is also detected in similar populations of neurons in the lumbar spinal cord. Based on

these data, a possible location for prion interference is within VMNs in the lumbar spinal cord ipsilateral to the site of inoculation.

In natural prion diseases with an infectious etiology, it is not known whether hosts are infected with prions a single time or if multiple prion infections occur. However, the mechanisms of prion adaptation following a single prion infection or multiple prion infections are likely similar, based on the following evidence. The interval between prion inoculation and the initial detection of prion replication, the "zero phase," can be extended by inoculation with a lower dose of prions (20, 21) (Fig. 7A). Once prion replication is detected, the rates of prion replication are similar between high and low doses of prions and the extension of the incubation period of the lower dose of prions compared to higher doses is due to an extension of the zero phase (20, 21) (Fig. 7A). In addition, the rate of prion replication and the accumulation of PrP<sup>Sc</sup> can differ between prion strains (25, 29) (Fig. 7A). The ability of the blocking strain to interfere with replication of a superinfecting prion strain is dependent on replication of the blocking strain in a location that is infected by both prion strains (2, 18, 35). The critical parameter for prion strain interference is not whether two prion strains are inoculated at the same time or separately but when and where prion replication occurs. For example, when a low dose of a quickly replicating short-incubation-period strain (Fig. 7B, fast strain) is inoculated at the same time as a high dose of a slowly replicating long-incubation-period strain (Fig. 7B, slow strain), the onset of prion replication of the slow strain occurs prior to replication of the fast strain (Fig. 7B). If common sites of infection are used by both strains, this situation can lead to an extension of the incubation period of the fast strain or a complete blockage of the fast strain by the slow strain (15). Similarly, the same relative time to onset of replication of the fast and slow strains can occur when a high dose of the slowly replicating long-incubation-period strain is inoculated first (Fig. 7C, slow strain) and at a later time point the host is infected (i.e., superinfection) with a high dose of a rapidly replicating short-incubation-period prion strain (Fig. 7C, fast strain). In this example, the slow strain could interfere with the fast strain when both strains were infected at the same time or when the slow strain was inoculated prior to superinfection of the animal with the fast strain.

Aside from gaining an understanding of the mechanism of prion adaptation, deciphering the mechanism of how a prion strain with a long incubation period and low pathogenicity can interfere with the replication of a short-incubation-period highly pathogenic strain to cause disease is of great interest. The ability to mimic this process in the absence of prion infection could reduce prion replication in the CNS and serve as a possible therapy.

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