

## Subclinical Scrapie Infection in a Resistant Species: Persistence, Replication, and Adaptation of Infectivity during Four Passages

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**Cross-species infection with transmissible spongiform encephalopathy agents may lead to subclinical infection and to adaptation of the infection to new species. This is of particular concern for the millions of people possibly exposed to bovine spongiform encephalopathy (BSE) by consumption of BSE-infected beef. Subclinical infection was studied by making 4 serial passages of hamster scrapie agent (263K) in mice. At each step, infectivity was followed by inoculation of hamsters and mice. Subclinical infection was demonstrated either by detection of abnormal protease-resistant prion protein (PrP-res) or in the absence of PrP-res by detection of infectivity. Replication and adaptation of hamster infectivity in mice was shown in year 2 after initial mouse passage. In third and fourth passages, dual-tropic, mouse-tropic, and hamster-tropic infectivity was found in different animals. In some cases infectivity similar to the original 263K hamster scrapie strain was found after 2 or 3 serial mouse passages totaling 1200–1550 days.**

Transmission of bovine spongiform encephalopathy (BSE) to humans in Europe has increased concern about the risk posed by transmissible spongiform encephalopathies (TSE). In addition to direct induction of clinical disease, cross-species TSE infection may result in a subclinical carrier infection that may subsequently evolve or adapt to a more virulent form. This possibility has led to concern about contamination of the blood supply and of surgical instruments by persons exposed to BSE. The situation is complicated by the possibility that BSE has spread to sheep in Europe [1]. There is no epidemiologic evidence for the transmission of sheep scrapie to humans, but nothing is known about the potential risk sheep-derived BSE may pose to humans or other species. Similarly, in the United States, the risk posed by chronic wasting disease (CWD) of elk and deer for humans, wildlife, and livestock is not clear [2]. Despite the lack of evidence for natural spread of CWD to cattle or humans, the possibility of infected asymptomatic carriers in animal or human populations is of concern.

Cross-species transmission of TSE infectivity leading to clinical disease has been observed in a variety of animal species. Usually the incubation period is longer in the first few passages but eventually stabilizes to a predictable value in the new host. Absence of cross-species transmission to host species that are not globally resistant to all TSE agents has been observed in only a few situations: BSE infection of hamsters [3], CWD infection of hamsters [4], transmissible mink encephalopathy

infection of mice [5], and hamster scrapie strain 263K infection of mice [6].

In these experiments, absence of transmission is usually based on the lack of clinical disease within the lifespan of the recipient, although in some cases lack of typical central nervous system pathologic changes have also been documented. Only in the hamster 263K mouse model has infectivity been directly assayed by inoculation back into hamsters to search for the existence of asymptomatic carrier mice. Some carrier mice were detected in the first passage, but not in second or third mouse passages, leading to the conclusion that the 263K infectivity detected in first-passage mice was merely the original inoculum and that replication had not occurred [7].

In previous experiments, we found that hamster 263K scrapie persisted in mouse brain and spleen for  $\leq 2$  years without causing clinical disease [8]. This persistence required the expression of the mouse prion protein (PrP) gene and implied that the foreign scrapie agent might have replicated since PrP was required and PrP is a susceptibility factor for scrapie replication. This possibility was also supported by experiments that analyzed infectivity in mice infected with 263K hamster scrapie in which 1 mouse had a significantly increased titer consistent with replication [9].

To define precisely the kinetics of replication that might occur after cross-species infection, we analyzed infectivity and protease-resistant PrP (PrP-res) in 23 mice with asymptomatic carrier infections at various times after infection with 263K hamster scrapie. Replication of infectivity was not detected in the first year; however, during year 2 we observed significant replication of hamster agent plus adaptation to a form virulent for mice [10]. In current studies of third and fourth passages in mice and hamsters, infectivity detected after 3 or 4 passages

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**Table 1.** Detection of protease-resistant prion protein (PrP-res) and clinical disease in first-passage mice.

Day	Clinical disease <sup>a</sup>	Hamster PrP-res <sup>b</sup>	Mouse PrP-res <sup>b</sup>
0.1	0/2	2/2	NT
5	0/2	0/2	0/2
20	0/2	0/2	0/2
60	0/2	0/2	0/2
130	0/2	0/2	0/2
240	0/2	0/2	0/2
310	0/2	0/2	1/2
463	0/3	0/3	0/3
574	0/16	0/16	7/16
693	0/13	0/13	3/13
782	0/2	0/2	2/2

NOTE. Primary C57BL/10 weanling mice were inoculated intracerebrally with 50  $\mu$ L of a 1% hamster brain suspension containing  $10^7$  ID<sub>50</sub> U of hamster scrapie strain 263K. Clinical disease refers to signs of clinical scrapie including somnolence, kyphosis, tremors, stilted gait, and ataxia. Mice with these signs usually progressed to a moribund status within 2 weeks. To assess clinical status mice were monitored daily by animal caretakers and weekly by laboratory staff.

<sup>a</sup> No. of animals with clinical scrapie/total.

<sup>b</sup> No. of animals with PrP-res/total. As described [10], hamster PrP-res was detected by immunoblot by using a hamster PrP-reactive monoclonal antibody (3F4) [11], which does not react with mouse PrP. Mouse PrP-res was detected by immunoblot in samples negative for hamster PrP-res by using a rabbit anti-PrP peptide serum (R30) [12].

had variable tropism for mice or hamsters or both. Hamster-tropic infectivity appeared to be similar to the 263K hamster scrapie agent originally inoculated, suggesting that hamster-tropic scrapie can replicate for extended periods in the presence of mouse PrP without losing its original hamster specificity.

## Results

In earlier experiments, mice were inoculated intracerebrally with hamster scrapie (strain 263K). For a 2-year period, no clinical signs were observed in any mice. At various times individual mice were sacrificed and brain homogenates were analyzed by Western blotting for PrP-res. Hamster PrP-res was detected only at 2 h after inoculation, and this appeared to be residual inoculum. Between days 5–240, mice were negative for PrP-res from either hamster or mouse. Starting at day 310, an occasional mouse had detectable mouse PrP-res (table 1); at day 782, the last time point studied, both mice were positive for mouse PrP-res.

To determine whether scrapie infectivity was present in the PrP-res–negative first-passage mice, brain homogenates from 2 PrP-res–negative donors at days 1–693 days after infection were analyzed by a second passage into mice and hamsters (table 2). No second-passage mouse developed clinical disease, although a significant percentage developed PrP-res by days 650–750. In contrast, hamsters inoculated with the same homogenates developed clinical disease. The incidence of disease was 100% at the earlier time points, less in donors at days 20–240, but again 100% at later time points (days 463–693; table 2). Thus, in PrP-res–negative clinically normal mice inoculated with 263K hamster scrapie up to 693 days earlier, there

was substantial scrapie infectivity with a preferential tropism for hamsters rather than mice. The lower incidence of disease seen with homogenates from donors sacrificed from days 20 to 240 suggested that an “eclipse” period after the early phase of sequestration of the infectivity was followed by a later stage of infectivity replication starting about 1 year after initial infection.

Randomly selected mice from the second passage were sacrificed 650–750 days after inoculation for a third passage into mice and hamsters. In some cases no infectivity was found, but in other mice significant infectivity was detected (table 3). Infectivity from some donors appeared to infect hamsters preferentially (see donor 1, day 574 first passage). In contrast, others showed infectivity with a strong preference for mice (see donor 1, day 693 first passage). Yet other donors appeared to have dual-tropic infectivity or a mixture of mouse and hamster tropic infectivity. These data suggest that in the course of replication in mice the original hamster scrapie strain 263K in some cases adapted to become more infectious for mice.

In the final series of experiments, some mice and hamsters from the third passage were used to make a fourth passage into mice and hamsters (table 4). For most mouse and hamster donors, infectivity still appeared to be dual tropic for both mice and hamsters. However, third-passage mouse donors from line 693/1 continued to exhibit only mouse tropism with an incubation period of about 120 days. This incubation period was similar in a fifth mouse passage (data not shown), suggesting the development of a new strain with a stable mouse-tropic phenotype. Analysis of glycoform ratios indicated a consistent unique pattern and further supported the conclusion that this was a relatively homogeneous strain (figure 1). For the other third-passage mice (lines 574/2 and 782/1), a fourth mouse passage gave longer incubation periods (about 300 days). By analysis of glycoform ratios, the 782/1 line had a unique pattern

**Table 2.** Incidence of clinical disease and protease-prion protein resistant (PrP-res) in hamsters and mice inoculated with brain homogenates from PrP-res–negative first-passage mice.

Days in first passage	% clinical disease		% PrP-res positive <sup>a</sup> mice
	Hamsters	Mice	
0.1	100	NT	NT
5	100	NT	NT
20	91–100	NT	NT
60	17–92	NT	NT
130	38–72	0	0
240	75–82	NT	NT
463	100	0	50
574	100	0	75
693	100	0	42
782	100	100	100

NOTE. Secondary-passage mice and hamsters were inoculated intracerebrally with 50  $\mu$ L of 1% brain suspension from primary mice sacrificed at indicated number of postinoculation days (dpi). For second-passage experiments, 2 PrP-res–negative first-passage donors were tested at selected time points, except at 782 dpi, where only 1 donor was used and the donor was PrP-res positive. Data were pooled for donors at each point. NT, not tested.

<sup>a</sup> Mice were sacrificed at 650–750 dpi for analysis of PrP-res.

**Table 3.** Analysis of third passage in hamsters and mice.

Time of first passage (days)	Second-passage donor mice		Third-passage recipients			
			Hamsters		Mice	
	No.	PrP-res	Clinical disease	Incubation period, days	Clinical disease	Incubation period, days
130	1	–	0/12	>600	0/12	>700
130	2	–	0/12	>700	0/12	>700
463	1	–	0/12	>700	0/12	>520
463	2	+	2/12	233–245	0/12	>700
574	1	+	12/12	215 ± 11	3/12	531
574	2	+	12/12	180 ± 2	10/10	314 ± 18
693	1	+	1/12	231	12/12	183 ± 22
782	1	+	12/12	197 ± 12	12/12	320 ± 10

NOTE. Incubation period was the time from inoculation until development of obvious clinical disease. Third-passage mice and hamsters were inoculated intracerebrally with 50  $\mu$ L of a 1% brain suspension from secondary mice sacrificed 650–750 days after inoculation. PrP, protease-resistant prion protein.

that differed from 693/1; however, the 574/2 line showed considerable heterogeneity, suggesting this was still a mixture of strains (figure 1).

For 3 third-passage hamster donors (574/1, 574/2, and 782/1), the incubation period in the fourth passage in hamsters was 80–85 days, suggesting similarity to the original 263K scrapie strain. In both third- and fourth-passage hamsters, the PrP-res banding pattern by Western blotting was indistinguishable from strain 263K (figure 2), further supporting the close relationship to the 263K strain. Nevertheless, mice inoculated with these same hamster brain homogenates developed clinical scrapie at 227–578 days (table 4), indicating that this infectivity was not identical to the 263K hamster scrapie strain or that it might be a mixture of strains.

## Discussion

In the experiments described, hamster-derived scrapie infectivity replicated in mice for 3 serial passages over nearly 4 years. These results contrast with those of Kimberlin et al. [7], who concluded that 263K hamster scrapie could not replicate in mice and that only the original inoculum could persist. Differences between study results may relate to the fact that in earlier studies mouse passages were done at days 319, 158, and 152, but in our studies infectivity for second-passage mice was not detected until after day 463 (table 2).

In our analysis there was no evidence of replication of hamster scrapie agent during the first year after inoculation. The original inoculum was detectable several hours after infection and steadily decreased over the following months. Only after day 463 was there evidence of an increase in infectivity compared with preceding study days (table 2) and at this point there was still no detectable PrP-res. Later there was evidence of further increases in infectivity as well as gradual adaptation

toward increasing virulence for mice. Thus, in these experiments, we identified two distinct phases in asymptomatic PrP-res–negative mice, a persistent phase followed by a replicative phase. PrP-res was not a reliable marker for either phase.

During the second mouse passage, two different scenarios were observed. Mice inoculated with homogenates from day 130 in the first passage had no PrP-res (table 2), and there was no detectable infectivity for either mice or hamsters (table 3). In contrast, mice inoculated with homogenates from later in the first passage developed PrP-res, and there was evidence of further adaptation of infectivity to mouse tropism (table 3). In fact, donor 693/1 appeared to have generated infectivity with preferential tropism for mice. The adaptation we observed was similar to that seen by Kimberlin and coworkers who used the same system [6, 7, 14].

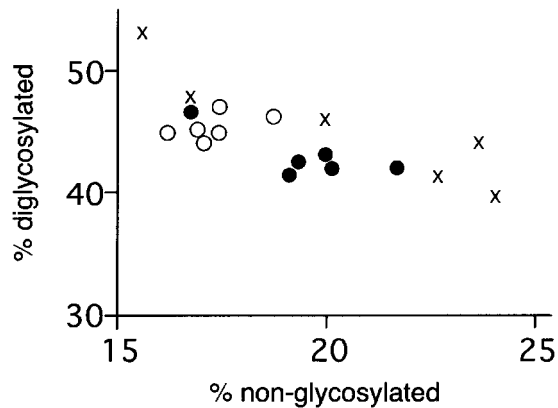
In the current experiments, both hamsters and mice from the third passage were passaged again in both species. In the third passage in mice, the infectivity detected was either exclusively mouse tropic or dual tropic. In contrast in the third passage in hamsters, infectivity was dual tropic, however, the incubation period was rapid (80–85 days) in hamsters, similar to 263K, and slow (227–578 days) in mice. Thus, this infectivity was either slightly different from 263K or a mixture of 263K plus a more mouse-tropic strain.

Although we noted adaptation of hamster scrapie to mice in the second year after infection (table 2), most of the infectivity in first-passage mice maintained its virulence for hamsters. Replication of this infectivity occurred despite the absence of protease-sensitive hamster PrP (PrP-sen). Furthermore, in second- and third-passage mice, hamster-tropic infectivity continued to persist and replicate. Thus, we detected infectivity with a close resemblance to hamster strain 263K after 2 or 3 mouse passages totaling 1200 to >1550 days. These data would be easy to explain if the agent was a conventional virus with a nucleic acid genome capable of mutation to allow adaptation to a new

**Table 4.** Results of fourth passage in mice and hamsters.

First-/second-passage mouse	Third-passage donor	Fourth-passage recipients			
		Hamsters		Mice	
		Clinical disease	Incubation period, days	Clinical disease	Incubation period, days
574/1	Hamster	4/4	85 ± 0	5/12	568 ± 23
574/2	Hamster	4/4	84 ± 1	9/11	578 ± 6
	Mouse 1	12/12	182 ± 16	10/10	294 ± 3
	Mouse 2	12/12	19 ± 72	11/11	313 ± 15
693/1	Mouse 1	0/8	>542	12/12	120 ± 2
	Mouse 2	0/8	>542	12/12	118 ± 3
782/1	Hamster	4/4	80 ± 2	10/12	227 ± 41
	Mouse 1	10/12	270 ± 57	12/12	286 ± 50
	Mouse 2	1/12	387	12/12	314 ± 16

NOTE. Incubation was the time from inoculation until development of obvious clinical disease. Fourth-passage mice and hamsters were inoculated intracerebrally with 50  $\mu$ L of a 1% brain suspension from third-passage mice sacrificed at the time of clinical disease (see table 3).



**Figure 1.** Glycoform ratios of resistant form of prion protein (PrP-res) from fourth-passage mice. Immunoblots were made with 1 mg of brain tissue per lane obtained from 20% brain homogenates digested with proteinase K as described [13]. Mouse PrP-res was detected by immunoblot in samples negative for hamster PrP-res by using a rabbit anti-PrP peptide serum (R30) [10]. Glycoform ratios were determined by plotting the percent of total PrP-res found in the diglycosylated PrP-res band vs. that found in the nonglycosylated PrP-res band. Data are from fourth-passage mice from the following donors: 574/2, third-passage mouse 1 and 2 (x); 782/1, third-passage mouse 1 and 2 (●); 693/1, third-passage mouse 1 and 2 (○).

species but are more difficult to reconcile with the protein-only hypothesis, where PrP-res might be the agent and the species tropism might be dependent on the species of the PrP used to generate the new PrP-res [15]. To remain consistent with this hypothesis, the incoming hamster PrP-res would have to “imprint” its unique structural properties on the new PrP-res generated from mouse PrP-sen during the replication that occurred over the years of these experiments [16–22]. More precise structural information on PrP-res will be required to either validate or exclude this possibility.

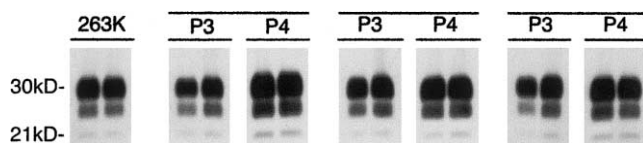
Possibly many TSE agents have the capacity to both persist and adapt over long periods. In wildlife and in agricultural settings where TSE diseases might be transferred across species barriers, there could be other situations that lead to subclinical infection and unexpected adaptation or spread to additional

species. For example, although sheep scrapie is thought to present no risk to humans, it may be the source of BSE in Europe [23, 24] and possibly of CWD in the United States. If BSE is derived from sheep scrapie, then adaptation during passage in cattle may have increased its pathogenicity for humans. A similar situation could occur with CWD. CWD transmission to other cervids or livestock could change its characteristics including its potential for transmission to humans.

Humans exposed to BSE-infected beef may be somewhat resistant to development of clinical variant Creutzfeldt-Jakob disease as evidenced by the low number of clinically diseased people compared with the number potentially infected. However, there is concern that a subclinical carrier state might occur in many asymptomatic persons. If true, the danger of replication, adaptation, and further spread of the agent from these people to others might increase at longer postexposure times. Furthermore, in the absence of precise information on the infectious dose of BSE for humans, it is impossible to predict the number of possible subclinical carriers in the population. Because of the low levels of agent expressed, such a subclinical state might only be detectable by transfer of infectivity and might escape detection by current biochemical methods. Therefore, subclinical human carriers might pose a serious risk for contamination of surgical instruments, tissue transplants, and blood products. It is important to be aware of these potential risks when designing policies to prevent further spread of BSE and other TSE diseases.

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**Figure 2.** Western blot analysis of protease-resistant prion protein (PrP-res) in brain tissue of third- (P3) and fourth-passage (P4) hamsters. *Left*, Positive brains from 2 hamsters infected with strain 263K are shown for comparison. Immunoblots were done as described in figure 1 except that hamster PrP-res was detected by using a hamster PrP-reactive monoclonal antibody (3F4) [11] that does not react with mouse PrP.

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