

## Prion distribution in hamster lung and brain following intraperitoneal inoculation

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**Prion titres were measured in the lungs and brains of Syrian hamsters after intraperitoneal inoculation with sucrose gradient-purified 263K prions ( $\sim 10^8$  LD<sub>50</sub>). Prions were detected in the lung of one hamster on day 7, but were not detected in the lungs of any other hamster until day 71. Prions were detected in the lungs of all hamsters sampled thereafter but titres remained low through day 127. Prions were first detected in the brain on day 35 and brain titres increased exponentially until day 127 with a doubling time of about 4.5 days. On day 133, titres averaged  $10^{8.0}$  LD<sub>50</sub>/g in brain and  $10^{5.0}$  LD<sub>50</sub>/g in lung. Two out of the five remaining hamsters were clinically normal but prion titres were not significantly different from those in the clinically affected hamsters. Thus, significant prion titres may be found outside the CNS in clinically normal hamsters.**

Prion diseases are fatal neurodegenerative diseases of animals and humans that result in accumulation in the brain of an abnormal host protein called prion protein (PrP) (Bolton *et al.*, 1982; Prusiner *et al.*, 1982*a*). Some human prion diseases are known to be caused by mutations in the gene (PRNP) that encodes the prion protein, while other human prion diseases are caused by infection with prions (Prusiner, 1993). Infection may cause most animal prion diseases but Parry has argued for a genetic cause as well (Parry, 1962, 1979). The fact that inherited prion diseases can be transmitted to animals by infection is a feature that is unique to this class of diseases (Prusiner, 1993).

A widely accepted theory for the nature of the causative agents, called prions (Prusiner, 1982), is based on the hypothesis first stated by Griffith (1967) that the causative agents are composed only of protein. The protein-only theory asserts that an abnormal host protein (called scrapie prion

protein or PrP<sup>Sc</sup>) causes prion diseases. PrP<sup>Sc</sup> differs from its normal counterpart (called the cellular prion protein or PrP<sup>C</sup>) by its conformation. Prion 'replication' occurs when the abnormal form, PrP<sup>Sc</sup>, binds to the normal prion protein and converts it to the abnormal conformation (Griffith, 1967; Bolton & Bendheim, 1988; Prusiner, 1991). Although the theory remains controversial, evidence in support of the protein-only model is substantial (Bolton *et al.*, 1982, 1987, 1991; McKinley *et al.*, 1983; Prusiner *et al.*, 1984; Hsiao *et al.*, 1989, 1990; Scott *et al.*, 1989, 1993; Meyer *et al.*, 1991; Büeler *et al.*, 1992).

Human prion diseases are rare regardless of their aetiology. In contrast, bovine spongiform encephalopathy (BSE) prions have infected a substantial number of cattle (Anderson *et al.*, 1996) and their apparent transmission to humans in the form of a new variant of Creutzfeldt–Jakob disease (nv-CJD) (Will *et al.*, 1996; Chazot *et al.*, 1996; Deslys *et al.*, 1997) raises concern for human health. While the evidence supporting a bovine origin for nv-CJD is compelling (Collinge *et al.*, 1996*b*; Almond & Pattison, 1997; Hill *et al.*, 1997; Bruce *et al.*, 1997), the exact route of infection and source of the contaminating prions are not clear. Acquisition of disease through contaminated food is a primary concern but other sources cannot be ruled out at this time. Infectious prions have not been detected in muscle from infected cattle (Bradley, 1993; Middleton & Barlow, 1993; Farquhar *et al.*, 1996) and meat is not considered the most likely source of prion contamination in the food supply. Instead, prions in neurological tissue (brain and spinal cord), added intentionally or unintentionally during the manufacture of meat products, are a more probable source of contamination (Chazot *et al.*, 1996; Collinge *et al.*, 1996*a, b*).

The possibility that nv-CJD is transmitted through pharmaceutical products manufactured directly from BSE-contaminated bovine tissues or with the use of other bovine-derived products contaminated by BSE prions should also be considered. The probability that a finished product would be contaminated depends on several factors, including the prevalence of BSE in the source herd, the tissues used, the concentration of prions in those tissues and the extent of inactivation or removal of prions during product manufacture. Thus, an understanding of the kinetics of prion replication or

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accumulation can be an important aspect in the selection of tissues for use in the manufacture of pharmaceutical products.

BSE prions have been detected only rarely in non-CNS tissues from clinical BSE cases (Bradley, 1993; Middleton & Barlow, 1993; Farquhar *et al.*, 1996; Wells *et al.*, 1998). In the largest reported study, more than 50 different tissues were sampled from experimentally infected cattle. Initially, infectivity was only detected in the distal ileum, where it was found from 6 months to 18 months post-inoculation, but not at 2 months (Wells *et al.*, 1996). Recently, Wells *et al.* (1998) reported that prion titre was found in the bone marrow of cattle killed at 38 months. This observation, coupled with the fact that bioassays are completed only for animals killed up to 22 months, suggests that additional tissues may harbour infectivity. In addition, interpretation of the negative bioassay results is made difficult by the species barrier, which decreases the sensitivity of the bioassay when the sample is from a different host species than the bioassay host (Barlow & Rennie, 1976; Manuelidis *et al.*, 1978; Kimberlin *et al.*, 1987; Prusiner *et al.*, 1990; Kocisko *et al.*, 1995; Wells *et al.*, 1998).

Prion titres outside the CNS can be significant in species other than bovines (Lavelle *et al.*, 1972; Kimberlin & Walker, 1979; Hadlow *et al.*, 1982; Casaccia *et al.*, 1989; Rubenstein *et al.*, 1991; Race & Ernst, 1992; Fraser, 1996). Prions are commonly found in spleen (Lavelle *et al.*, 1972; Hadlow *et al.*, 1974, 1982; Kimberlin & Walker, 1979; Rubenstein *et al.*, 1991; Race & Ernst, 1992; Fraser, 1996) and lymphatic tissues (Hadlow *et al.*, 1974, 1982; Fraser, 1996) but have been detected occasionally in intestine (Hadlow *et al.*, 1974, 1982), blood (Casaccia *et al.*, 1989) and other tissues (Hadlow *et al.*, 1974, 1987; Sakaguchi *et al.*, 1993). Studies of prion replication or accumulation in extraneural tissues are not common, however, and therefore this study was undertaken to compare prion titres in lung with those in brain following peripheral infection. Lung was chosen because PrP<sup>C</sup> is known to be expressed in this tissue (Bendheim *et al.*, 1992), it is an ingredient in pet foods and it is used in the manufacture of some medicines (e.g. surfactant). The hamster 263K scrapie prion model was chosen because it offers a well-characterized model of prion disease with a sensitive bioassay.

Thirty-four Syrian hamsters (virus-free weanling females) were inoculated in the peritoneal cavity with 50 µl 263K scrapie prions (1:100 dilution), purified as described previously (Bolton *et al.*, 1987; Bendheim *et al.*, 1988). Three hamsters were humanely killed by CO<sub>2</sub> asphyxiation on each of the days indicated in Fig. 1. The lungs were aseptically removed and immediately frozen, and then the brains were aseptically removed and frozen. This order was observed to eliminate the possibility that brain tissue would contaminate the lung samples. All tissue samples were stored at -70 °C until processed for bioassay. Homogenates (10% by weight) were made in PBS from a sample of each tissue using a motorized bladed tissue homogenizer. All lung samples for each time-point were processed before the brain samples and the

homogenizer was thoroughly rinsed between each tissue. The homogenizer blades were decontaminated by first running them in 1 M NaOH followed by an overnight soaking in an excess of 1 M NaOH. The blades were then washed, rinsed with distilled water and autoclaved at 15 p.s.i. for 20 min between each set of time-points. Each homogenate was sedimented at 1000 g for 10 min and the supernatant was dispensed in three aliquots into virgin glass vials previously sterilized by baking overnight at 450 °C. Each vial was capped and stored at -70 °C until the contents were bioassayed.

Samples, as specified above, were inoculated into the right cerebral hemisphere of five or six ~35-day-old hamsters, (25 µl per hamster). The animals were observed 5 days per week (Monday to Friday) for clinical signs of scrapie from about day 54 after inoculation until approximately day 120, and then three times per week (Monday, Wednesday and Friday) until the assay was terminated. Sample titres were calculated using a mathematical relationship between the times from inoculation to the onset of clinical disease and to death (Prusiner *et al.*, 1982*b*). Some bioassay samples produced a less than 100% response in the assay animals (i.e. some hamsters remained normal at the end of the bioassay or died after 200 days without showing clinical signs of scrapie). In those cases, the titres were calculated based on the Poisson distribution  $X_{(n)}/N = e^{-m}$ , where  $X_{(n)}$  is the number of hamsters free of disease,  $N$  is the total number of hamsters inoculated, and  $m$  is the number of infectious units per inoculum. The asterisks in Fig. 1 indicate those time-points at which some samples did not cause clinical scrapie in any of the bioassay animals inoculated. In those cases, the maximum possible sample titre that could have gone undetected in the bioassay was calculated using the Poisson distribution formula with the assumption that the  $N + 1$  hamster would have contracted the disease.

Prions were detected in the day 7 lung sample from one hamster (two out of six bioassay hamsters affected), but not in either of the other two hamsters on day 7 (none of the twelve bioassay hamsters) or in any of the lung samples from days 21, 35 and 49 (Table 1). For those time-points, the values plotted in Fig. 1 indicate the maximum titre that could have gone undetected in this bioassay as described above. Prions were detected in all of the lung samples from the hamsters examined on days 71–133 but titres remained low until the last time-point (Fig. 1; Table 1).

Prions were not detected in the brain until day 35 when they were found in brain homogenates from two out of three hamsters sampled (Fig. 1; Table 1). Titres in the brain increased exponentially from day 35 and appeared to peak near the end of the clinical phase at day 127 with an average titre of  $10^{9.2}$  LD<sub>50</sub>/g. The rate of increase in prion titre from day 35 to day 127 was equal to a doubling time of 4.5 days. This rate is substantially slower than the doubling time of 2.8 days for replication of the 263K agent in brain following intracranial inoculation (Bolton *et al.*, 1991). Thus, the increased incubation period observed in hamsters infected with 263K scrapie prions

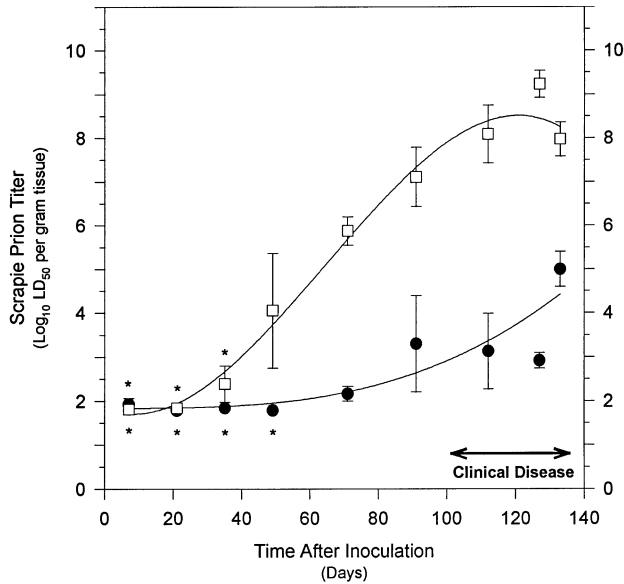


Fig. 1. Prion titres in hamster lung and brain following intraperitoneal inoculation. □, Average brain titre; ●, average lung titre. The double arrow indicates the approximate period during which hamsters exhibited clinical signs of disease. Asterisks denote time-points in which some samples did not cause disease in any of the bioassay animals (see text and Table 1). The lines were calculated as third-order linear regressions using the least-squares method. The error bars represent the standard error of the mean. The large standard errors in the brain samples on day 49 and the lung samples on day 91 are due to high prion titres in only one of the three hamsters from each sample.

by the intraperitoneal route appears to be due to a combination of a longer initial lag phase and slower replication rate than in brain (Bolton *et al.*, 1991).

Two of the five hamsters sampled on day 133 were clinically normal. At that time, the prion titres measured in the brains and lungs of the normal hamsters were not significantly different from those of the three clinically affected hamsters (Table 2).

Table 2. Prion titres in lung and brain of normal and diseased hamsters

Clinical status	Prion titre at 133 days (LD <sub>50</sub> /g) ± SD	
	Lung	Brain
Diseased	4.8 ± 1.1	8.0 ± 1.1
Normal	5.3 ± 0.7	7.9 ± 0.8
All animals	5.0 ± 0.9	8.0 ± 0.9

In this study, prion titres in the lung remained undetectable or at very low levels until near the end of the disease course even as the brain titres rose exponentially. One interpretation of this result is that disruption of the blood/brain barrier during the disease course (Chung *et al.*, 1995; Vorbrodth *et al.*, 1997) allows prions to enter the blood stream and accumulate in the lung.

The importance of understanding the distribution of prions in non-CNS tissue throughout the course of infection is made clear by the apparent aetiological association between BSE prions and nv-CJD. The results presented herein indicate that clinically normal animals in the latter stage of the incubation period can contain significant prion titres in non-CNS tissue. However, the potential significance of these results for human health must be evaluated within the proper context.

Studies in animal model systems have several distinct advantages over studies of the natural disease: a sensitive bioassay, controlled conditions of infection, serial sampling throughout disease, and better statistical power due to a larger sample size. The hamster 263K model provides a particularly sensitive system to detect low concentrations of prions, with the ability to readily detect prions over a range of < 10<sup>2</sup> to >

Table 1. Proportion of hamsters showing clinical signs of scrapie

	Time after inoculation (days)								
	7	21	35	49	71	91	112	127	133
<b>Lung</b>									
Time-course*	1/3	0/3	0/3	0/3	3/3	3/3	3/3	3/3	3/5
Bioassay†	2/18	0/18	0/16	0/18	6/18	12/18	11/18	15/18	30/30
<b>Brain</b>									
Time-course*	0/3	0/3	2/3	3/3	3/3	3/3	3/3	3/3	5/5
Bioassay†	0/17	0/16	8/18	15/18	18/18	18/18	18/18	18/18	30/30

\* No. of intraperitoneally inoculated hamsters with detectable scrapie in the tissue sample/total no. of intraperitoneally inoculated hamsters sampled on that day.

† No. of bioassay hamsters with scrapie/total no. of bioassay hamsters inoculated with those samples.

$10^{9.5}$  LD<sub>50</sub>/g. Other systems are much less sensitive, so that negative results must be interpreted with caution. One could argue that direct bioassay of BSE prions in cattle provides the best system for ascertaining titres in bovine tissues but the expense required to achieve the statistical power of an animal model makes this approach impractical. The alternative bioassay system, inoculation of BSE prions from bovine tissues into mice, may be as much as 1000-fold less sensitive than if the measurements were made in cattle (Fraser *et al.*, 1992; Bruce *et al.*, 1994; Wells *et al.*, 1998). Scott *et al.* (1997) recently described construction of transgenic mice that possess a bovine PrP transgene but lack the mouse PrP gene. The mice are uniformly susceptible to infection with bovine prions after an incubation period of ~ 200 days. Bioassays using those or similar mice should enable more accurate determinations of prion titres in bovine tissues.

Three other factors should be considered when interpreting the results presented here. First, hamster 263K scrapie prions may behave differently from BSE prions. Second, the pathophysiology of prion disease may be significantly different in hamsters compared with bovines or other species. Third, infection by intraperitoneal inoculation may not produce the same pattern of extraneural prion replication or accumulation as the natural (oral) route of infection in BSE.

Clearly, two possibilities exist to explain the failure to detect prions in non-CNS tissues from BSE-affected cattle: either the prions are not present in the tissues or the detection systems used are insufficiently sensitive. If the former case is correct, there is no risk to human health from these tissues. If the latter is true, the risk, although it may be very low, remains unknown. The results presented in this report strengthen the latter hypothesis and argue that tissues from younger animals should be used in the manufacture of pharmaceutical products in preference to those from older animals where possible. Tissues from younger animals would be less likely to contain significant prion contamination because they would have been incubating the disease for a shorter time. Furthermore, the data presented herein indicate that measurements of prion titres in extraneural tissues from natural prion diseases should be interpreted with caution in light of the potential for false negative results due to a combination of low prion titres and a relatively insensitive bioassay.

The excellent technical assistance of Ms Michelle Gonzalez and Ms Maya Nadolczak is gratefully acknowledged. The New York State Office of Mental Retardation and Developmental Disabilities supported this research. The use of animals in this research was reviewed and approved by the Institutional Animal Welfare Committee at the Institute for Basic Research.

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Received 6 January 1998; Accepted 17 June 1998