

High Incidence of Scrapie Induced by Repeated Injections of Subinfectious Prion Doses†

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To clarify the mechanisms leading to the development of Creutzfeldt-Jakob disease in some recipients of pituitary-derived human growth hormone (hGH), we investigated the effects of repeated injections of low prion doses in mice. The injections were performed, as in hGH-treated children, by a peripheral route at short intervals and for an extended period. Twelve groups of 24 mice were intraperitoneally inoculated one, two, or five times per week for 200 days with 2×10^{-5} to 2×10^{-8} dilutions of brain homogenate containing the mouse-adapted C506M3 scrapie strain. Sixteen control mice were injected once a week for 200 days with a 2×10^{-4} dilution of normal brain homogenate. Of mice injected in a single challenge with a scrapie inoculum of a 2×10^{-4} , 2×10^{-5} , or 2×10^{-6} dilution, 2/10, 1/10, and 0/10 animals developed scrapie, respectively. Control mice remained healthy. One hundred thirty-five of 135 mice injected with repeated prion doses of a 2×10^{-5} or 2×10^{-6} dilution succumbed to scrapie. Of mice injected with repeated scrapie doses of a 2×10^{-7} or 2×10^{-8} dilution, 52/59 and 38/67 animals died of scrapie, respectively. A high incidence of scrapie was observed in mice receiving repeated doses at low infectivity, whereas there was no disease in mice that were injected once with the same doses. Repeated injections of low prion doses thus constitute a risk for development of prion disease even if the same total dose inoculated in a single challenge does not induce the disease.

Creutzfeldt-Jakob disease (CJD) is a rare transmissible dementia due to unconventional pathogenic agents called prions. It belongs to the group of transmissible spongiform encephalopathies, which also comprises scrapie in sheep and bovine spongiform encephalopathy in cattle. CJD occurs as a sporadic, familial, or iatrogenic form (iCJD). Since 1985, more than 160 cases of CJD transmitted iatrogenically by pituitary human growth hormone (hGH) have been described in a number of countries, including France, the United States, and the United Kingdom, with the majority of cases occurring in France (5, 24). Since 1985, urea-treated cadaveric hGH or recombinant hGH produced in *Escherichia coli* has been used in place of native pituitary-derived hGH. With urea-treated or recombinant hGH, no cases of iCJD have been identified. iCJD cases begin with a cerebellar syndrome without signs of dementia. The mean incubation period is 12 years, with a range of 2 years 3 months to over 30 years (4, 19, 21). The concentration of CJD prions within infected human pituitaries is unknown. hGH lots were probably contaminated at a low level with human prions, as experimental transmission of CJD to only one of the three squirrel monkeys inoculated with 1 of 76 potentially contaminated lots of hGH occurred after an incubation period of more than 5.5 years (11).

Homozygosity at codon 129 of the prion protein (PrP) gene is considered a risk factor for the development of iCJD after receiving pituitary-derived hGH (4). The high incidence of

CJD in pituitary-derived GH recipients could be related to a potentially higher susceptibility to prions in the young, as in variant CJD related to bovine spongiform encephalopathy (2). It could also be due to repeated injections of low doses of prions. We focus here on the latter aspect. The hGH treatments were administered weekly, at 10 to 20 IU (5 to 10 mg), given in two, three, five, or even seven intramuscular or subcutaneous injections (7, 21). The mean duration of hGH treatment was 6.4 years (range, 1 to 13 years) in France, similar to those in other countries (4). It is hypothesized that repeated injections of subinfectious prion doses might cause the development of transmissible spongiform encephalopathy. Indeed, a higher scrapie incidence was reported in repeated oral infection of hamsters with scrapie compared to single doses (9, 10, 12). The risk of infection was higher when the time intervals between repetitive doses were short (10). As no data are available on the effect of repeated peripheral injection of subinfectious prion doses, we investigated multiple injections of low prion doses in mice. The injections were performed, as in hGH-treated children, by a peripheral route at short intervals and for an extended period. Mice were injected by the intraperitoneal route, which constitutes one of the fastest peripheral routes (14).

MATERIALS AND METHODS

Animals. C57BL/6 mice at 6 weeks of age (Janvier, France) were used for this study. All animals were housed in level 3 care facilities of the Pasteur Institute, officially registered for prion experimental studies of rodents (Ministry agreement number A 75-15-27 for animal care facilities; agreement number 75-585 for animal experimentation).

Infectious material. The mouse-adapted scrapie strain C506M3 was stabilized and propagated in the C57BL/6 mouse line (17). The inoculum was a brain homogenate at 10% (wt/vol) in 5% glucose solution from a mouse with scrapie

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† In memory of Professor Dominique Dormont.

TABLE 1. Effects of repeated intraperitoneal infection of mice with scrapie

Inoculum		Parameter	Value					
Dilution	Tissue ^a (mg)		Scrapie (single injection)	Normal (injection/wk for 7 mo: 28 injections)	Scrapie			
					1 injection/wk for 7 mo: 28 injections	2 injections/wk for 7 mo: 57 injections	5 injections/wk for 7 mo: 143 injections	
2×10^{-4}	2.5×10^{-2}	Cumulative dose (mg)	2.5×10^{-2}	7×10^{-1}	7×10^{-1}	1.4	3.6	
		Transmission rate	2/10	0/16	Not done	Not done	Not done	
		Survival time ^b	421, 499					
2×10^{-5}	2.5×10^{-3}	Cumulative dose (mg)	2.5×10^{-3}		7×10^{-2}	1.4×10^{-1}	3.6×10^{-1}	
		Transmission rate	1/10		24/24	24/24	18/18	
		Survival time ^b	357		364 ± 19	339 ± 20	310 ± 21	
2×10^{-6}	2.5×10^{-4}	Cumulative dose (mg)	2.5×10^{-4}		7×10^{-3}	1.4×10^{-2}	3.6×10^{-2}	
		Transmission rate	0/11		24/24	22/22	23/23	
		Survival time ^b	>650		440 ± 70	368 ± 58	366 ± 51	
2×10^{-7}	2.5×10^{-5}	Cumulative dose (mg)	2.5×10^{-5}		7×10^{-4}	1.4×10^{-3}	3.6×10^{-3}	
		Transmission rate	Not done		14/18	19/19	19/22	
		Survival time ^b			456 ± 72	490 ± 56	364 ± 32	
2×10^{-8}	2.5×10^{-6}	Cumulative dose (mg)	2.5×10^{-6}		7×10^{-5}	1.4×10^{-4}	3.6×10^{-4}	
		Transmission rate	Not done		9/21	8/23	21/23	
		Survival time ^b			467 ± 57	450 ± 58	482 ± 61	

^a Amount of brain from which a single dose of inoculum was prepared.

^b Mean \pm SD (days).

at the terminal stage of disease, routinely titrating 5×10^8 to 5×10^9 50% lethal doses (LD₅₀) per gram in intracerebral-infection challenges (8, 15). The mean survival time was around 173 ± 5 (standard deviation [SD]) days after intracerebral infection or 333 ± 7 (SD) days after intraperitoneal challenge (8, 17). Healthy mouse brain homogenate was used for the negative controls.

Procedures. Twelve groups of 24 mice were inoculated by the intraperitoneal route one, two, or five times per week for 200 days with the mouse-adapted C506M3 scrapie strain. The inoculations were performed every Monday for the 1-injection-per-week schedule, every Monday and Wednesday for the 2-injections-per-week schedule, and every day from Monday to Friday for the 5-injections-per-week schedule. The injected prion doses (50 μ l) ranged from 10^{-5} (2.5×10^{-3} mg of brain) to 10^{-8} dilutions of the scrapie brain homogenate used as the inoculum. Three groups of 10 to 11 mice were injected once with scrapie inoculum dilutions of 2×10^{-4} , 2×10^{-5} , and 2×10^{-6} , respectively. Sixteen control mice received by the same route a 2×10^{-4} dilution of normal brain homogenate once a week for 200 days.

Scrapie diagnosis. Animals were examined three times per week for clinical signs of scrapie, which could include bradykinesia, waddling gait, poor coat condition, terminal incoordination, and weight loss. Scrapie-infected mice, animals affected by infection or injuries other than scrapie, and mice that had not developed disease 650 days after the first inoculation were euthanized by cervical dislocation at the terminal stage of the disease, following the Pasteur Institute's animal experimentation regulations.

Scrapie diagnosis was confirmed by detection of the pathological PrP scrapie (PrPsc) form, after proteinase K digestion and enzymatic deglycosylation with *N*-glycosidase F, by immunoblotting techniques, as previously described (16, 20). Briefly, tissue homogenates (5% [wt/vol] in 5% glucose) were treated or not with 10 μ g/ml of proteinase K (Sigma) for 1 h at 37°C. After *N*-glycosidase F deglycosylation (New England Biolabs), 2 \times Laemmli buffer was added, and 20 μ l of each extract, corresponding to 100 μ g of brain, was used for PrPsc detection. Samples were submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 12% polyacrylamide gels and electroblotted onto nitrocellulose membranes. Immunodetection was done using a pool of mouse monoclonal antibodies (SAF 32, raised against hamster PrP, which recognizes the octorepeat region located in the N-terminal part of PrP [1 μ g/ml], and SAF 84, raised against hamster PrP, recognizing an epitope located between 160 and 170 residues [1 μ g/ml]; both antibodies from Spi-Bio, France), followed by a peroxidase-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories). Immunoreactivity was visualized by chemiluminescence (enhanced chemiluminescence; Pierce, France). The survival period was calculated as the interval between the first inoculation and death or the extremis stage. The scrapie incidence and the mean survival time were determined for each group of mice, and the results were statistically analyzed using StatXact-4 software (Cytel Software Corp.).

RESULTS

The effect of intraperitoneal injections of repeated subinfectious doses of the C506M3 scrapie strain was investigated in

mice. Our results are summarized in Table 1. Figure 1 shows a representative scrapie diagnosis by proteinase K digestion of brain samples. None of the 16 mice injected with normal brain homogenate developed scrapie, indicating the absence of any background laboratory contamination. Of mice injected in a single challenge with a scrapie inoculum of a 2×10^{-4} , 2×10^{-5} , or 2×10^{-6} dilution, only 2/10, 1/10, and 0/10 animals developed scrapie. Animals that did not show any clinical signs of scrapie were all negative for the presence of PrPsc. Compared to the 5×10^8 to 5×10^9 mean LD₅₀ per gram in intracerebral-infection challenges, these results indicate that intraperitoneal injection of the C506M3 scrapie strain is 10^3 to 10^4 times less efficient than intracerebral inoculation (8, 15). These data are very similar to previous findings with other mouse-adapted scrapie strains (14). While a single injection of a 2×10^{-5} dilution scrapie dose induced scrapie in 10% of the injected animals, repeated injections of the same dose led to a 100% transmission rate for scrapie (Table 1). Of mice injected with a scrapie inoculum of a 2×10^{-5} dilution one, two, and five times per week for 200 days, 24/24, 24/24, and 18/18 animals died of scrapie, respectively. The high number of injec-

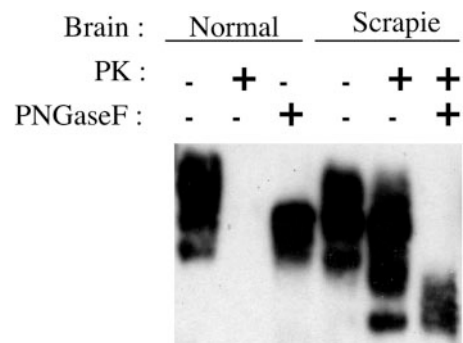


FIG. 1. Western blot diagnosis after proteinase K (PK) digestion; 0.1 mg of brain was plotted in each lane. Detection of PrP was done with SAF 32 and SAF 84 antibodies as described in Materials and Methods. +, present; -, absent.

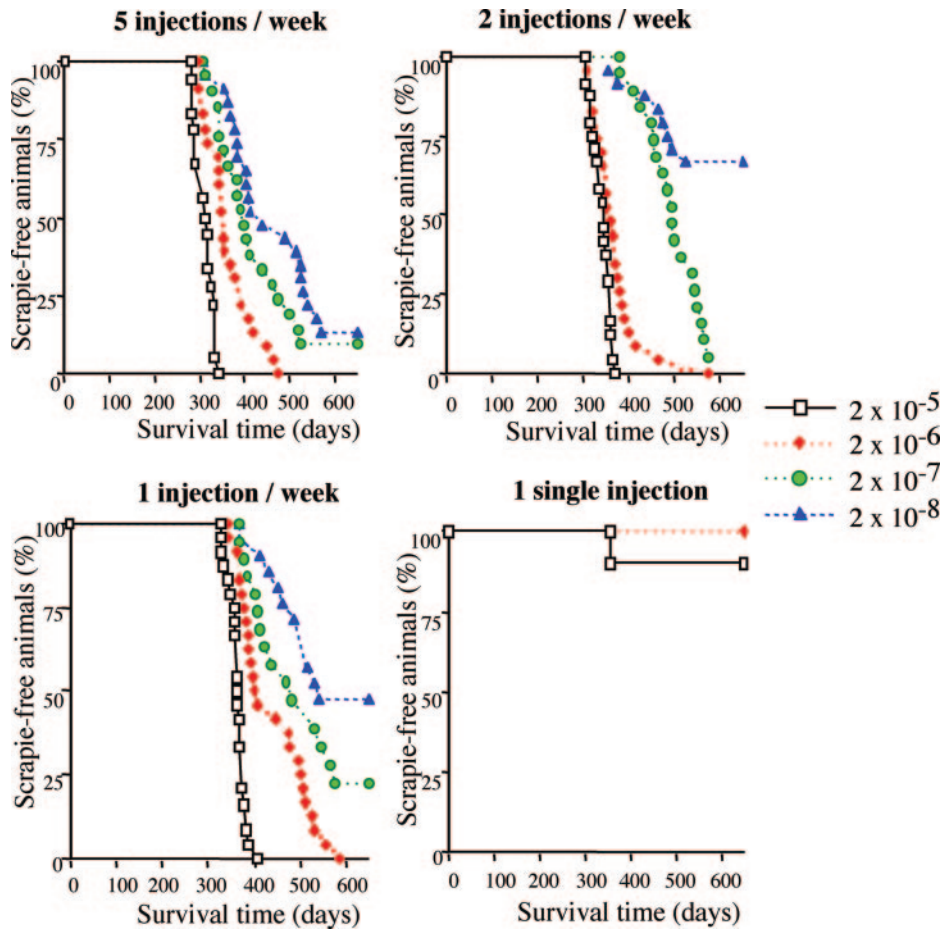


FIG. 2. Survival curves of mice intraperitoneally infected with different dilutions of scrapie inoculum. Twelve groups of 24 mice were inoculated one, two, or five times per week for 200 days with 2×10^{-5} to 2×10^{-8} dilutions of brain homogenate containing the mouse-adapted C506M3 scrapie strain. Sixteen control mice were injected once a week for 200 days with a 2×10^{-4} dilution of normal brain homogenate and remained healthy. Three groups of 10 mice received a single injection of a 2×10^{-4} , 2×10^{-5} , or 2×10^{-6} dilution of scrapie inoculum.

tions induced a significant decrease in the survival period, as the survival times for 28, 57, and 143 injections were 364 ± 19 , 339 ± 20 , and 310 ± 21 (SD) days, respectively ($P = 0.0001$ for 57 injections versus 28 injections; $P < 0.0001$ for 143 versus 28 injections; permutation test).

For increased dilutions of the infectious material, repeated injections resulted in a high incidence of scrapie, while the same doses inoculated in a single challenge did not induce the disease. Thus, all mice survived after injection of 2.5×10^{-4} mg extract in a single dose while 21/23 animals developed the disease for 3.6×10^{-4} mg extract injected at 2.5×10^{-6} mg five times a week (Table 1). Increasing the dilution of the scrapie inoculum was associated with both a progressive decrease of the infection rate and a significant increase of the survival period. Of mice injected with a scrapie inoculum at 2×10^{-6} dilution one, two, and five times per week for 200 days, 24/24, 22/22, and 23/23 animals died of scrapie, respectively. Of mice injected with repeated scrapie doses of a 2×10^{-7} or 2×10^{-8} dilution, 52/59 and 38/67 animals succumbed to scrapie, respectively. Mice without clinical signs of scrapie were all negative for the presence of PrPsc. As shown in Fig. 2, the survival time was significantly shorter when the prion dose was high.

For instance, the survival time for one injection per week was 364 ± 19 days for the 2×10^{-5} dose dilution and 467 ± 57 (SD) days for the 2×10^{-8} dose dilution, respectively ($P = 0.003$; permutation test). Moreover, the scrapie incidence was significantly higher when the intervals between injections were shorter. As shown in Fig. 2, the survival time was also shorter when the number of inoculations was high. For instance, the survival time for a 2×10^{-7} dilution of infective material was 456 ± 72 days for 28 injections (1 injection per week) and 364 ± 32 (SD) days for 143 injections (5 injections per week), respectively ($P = 0.0357$; permutation test).

For all doses given, we found that the incidence of scrapie was higher when the total dose was injected as multiple challenges than when it was injected as a single challenge. For instance, scrapie was detected in 1/10 mice for a single injection at the cumulative dose of 2.5×10^{-3} mg of inoculum brain, in 9/21 mice for 28 injections (1 injection per week for 7 months) at a cumulative dose of 7×10^{-5} mg, in 8/23 mice for 57 injections (2 injections per week for 7 months) at a cumulative dose of 1.4×10^{-4} mg, and in 21/23 mice for 143 injections (5 injections per week for 7 months) at a cumulative dose of 3.6×10^{-4} mg ($P < 0.0001$ for 143 injections versus a

single injection; $P = 0.0006$ for 143 injections versus 28 injections; $P = 0.0001$ for 143 injections versus 57 injections; Fisher's exact test).

DISCUSSION

The high incidence of CJD in recipients of pituitary-derived hGH suggests a role of repeated injections of low prion doses in the development of this lethal disease. As no data were available on repeated low-prion-dose inoculations by injection, our aim was to evaluate the cumulative risk in an animal model and possibly to identify the number of PrPsc molecules involved in the spread of infectivity. Here, we show that a high incidence of scrapie cases occurred in mice receiving repeated low doses of prion material, whereas there was no disease in mice that were injected once with the same total dose. The risk of infection was clearly related to the level of exposure to prions, defined as the dose, the number of injections, and the interval between inoculations. Our findings are consistent with those of Diringer et al., who also reported a higher scrapie incidence in repeated oral infection of hamsters with scrapie compared to using a single dose (10). However, our results show a greater effect on scrapie incidence, which might be due to the route of administration. Comparison of data shows that repeated intraperitoneal infection is 10^3 - to 10^4 -fold more effective than repeated oral infection. Interestingly, in agreement with the report that a single intraperitoneal dose is more efficient than an oral dose, our data indicate that the intraperitoneal route is probably more efficient than the oral route when using multiple doses (14). The risk of infection thus seems to be higher by injection of low prion doses than by ingestion of the same doses. The higher scrapie incidence in multiple intraperitoneal infection could also be related to the longer duration of prion administration in our experiment. We repeatedly treated mice with low-dose inocula for 7 months, whereas in Diringer's study (10), repeated oral infection lasted only 10 days.

A single injection of very low doses of prions, such as 2.5×10^{-4} mg of scrapie brain, did not lead to scrapie infection, which might reflect active or passive elimination of prions by the organism. Inoculated animals without clinical signs of scrapie were all negative for the presence of PrPsc, which suggests that a low-dose inoculum failed to induce a chronic subclinical prion disease in our mouse scrapie model. Subclinical scrapie infection was described in some low-dose infections with scrapie (13). It was characterized by high levels of infectivity and PrPsc without development of clinical signs of prion disease during a normal life span. Taylor et al. have reported intermittent mild or early clinical signs in mice inoculated with low doses of ME7 prion inoculum (22). Similarly, Thackray et al. have shown high levels of infectivity and PrPsc in PrP transgenic mice with subclinical disease following low-dose inoculation of ME7 or RML scrapie strains (23). The difference between these observations and our results is probably due to the scrapie and mouse strains and to the route of inoculation. In a single intracerebral injection, one LD_{50} unit contains about 10^5 PrPsc molecules in a hamster scrapie model (3). Our data suggest that intraperitoneal injection of a scrapie strain is 10^3 to 10^4 times less efficient than intracerebral inoculation. In a single intraperitoneal injection, one LD_{50} unit is

thus likely to contain about 10^8 to 10^9 PrPsc molecules. At low-level infectivity, PrPsc clearance may prevent infection. Here, we found that multiple injections of doses of $<10^{-4}$ LD_{50} may induce infection, and so repeated injections of only about 10^4 to 10^9 molecules of PrPsc (2.5×10^{-6} mg of scrapie brain tissue) seem to constitute a risk of infection.

We have found that the more frequent the injections, the higher the scrapie incidence. This suggests that after intraperitoneal inoculation, active or passive degradation of prions in the peritoneal cavity might be saturated when the intervals between injections are shorter. There is *in vivo* and *in vitro* evidence that macrophages and dendritic cells are involved in active prion degradation. *In vitro*, $CD11c^+$ myeloid dendritic cells rapidly processed and degraded PrPsc (18). Incubation of scrapie brain homogenate with peritoneal macrophages greatly decreased prion infectivity, suggesting that macrophages can inactivate the scrapie agent *in vitro* (6). *In vivo*, administration of dichloromethylene disphonate before scrapie infection leads to macrophage depletion and accelerated scrapie, suggesting that macrophages may eliminate a fraction of the prion inoculum (1). Thus, macrophages and dendritic cells are likely to be involved in prion clearance after peripheral inoculation.

The scrapie incidence could depend on both the probability of infection at each given injection and the cumulative prion effect. In the cumulative prion effect hypothesis, the risk of infection could be determined by the total dose, accumulating with each challenge. Our results suggest a cumulative effect of prions, as we found that the incidence of scrapie was significantly higher when the same total dose was delivered through several injections rather than all at once. Our findings differ from Diringer's experiment, where a total fixed dose had a significantly reduced probability of causing oral infection if the material was presented as multiple challenges (10). Gravenor et al. have established statistical models to quantify the magnitude of the effect of repeated doses of prions over prolonged periods (12). They showed, by analyzing Diringer's data, that although the risk of oral infection increases with repeated doses, it does so to a lesser degree than would be expected if challenges combine independently or in a cumulative manner. A detailed statistical study of our results is in progress using similar statistical models.

In conclusion, our data highlight possible infection mechanisms leading to development of prion diseases in iatrogenic exposures to very low prion contaminations. A high incidence of scrapie cases was observed in mice receiving repeated doses at low infectivity, whereas there was no disease in mice that were injected once with the same doses. Our findings constitute the first evidence that repeated injections of low prion doses constitute a risk for development of a prion disease even if the same total dose inoculated in a single challenge does not induce the disease. Multiple injections of subinfectious prion doses may thus be a potential hazard in vaccinations and repeated treatments, as has been the case in the past with pituitary-derived hGH.

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