Chronic subclinical prion disease induced by low-dose inoculum

Thackray, A M ; Klein, M A ; Aguzzi, A ; Bujdoso, R

DOI: https://doi.org/10.1128/jvi.76.5.2510-2517.2002

JOURNAL OF VIROLOGY, Mar. 2002, p. 2510–2517 Vol. 76, No. 5
0022-538X/02/$04.00+0 DOI: 10.1128/JVI.76.5.2510–2517.2002

Chronic Subclinical Prion Disease Induced by Low-Dose Inoculum

Alana M. Thackray,[1] Michael A. Klein,[2] Adriano Aguzzi,[3] and Raymond Bujdoso[1]*

Centre for Veterinary Science, Department of Clinical Veterinary Medicine, University of Cambridge, Cambridge CB3 0ES, United Kingdom,[1] and University Hospital Basel, Institute of Pathology, CH-4003 Basel,[2] and Institute of Neuropathology, University Hospital of Zürich, CH-8091 Zürich,[3] Switzerland

Received 24 September 2001/Accepted 16 November 2001

We have compared the transmission characteristics of the two mouse-adapted scrapie isolates, ME7 and Rocky Mountain Laboratory (RML), in tga20 mice. These mice express elevated levels of PrP protein compared to wild-type mice and display a relatively short disease incubation period following intracerebral prion inoculation. Terminal prion disease in tga20 mice induced by ME7 or RML was characterized by a distinct pattern of clinical signs and different incubation times. High-dose RML inoculated intracerebrally into tga20 mice induced the most rapid onset of clinical signs, with mice succumbing to terminal disease after only 58 ± 3 days. In contrast, high-dose ME7 gave a mean time to terminal disease of 74 ± 0 days. Histological examination of brain sections from prion-inoculated tga20 mice at terminal disease showed that ME7 gave rise to a more general and extensive pattern of vacuolation than RML. Low-dose inoculum failed to induce terminal disease but did cause preclinical symptoms, including the appearance of reversible clinical signs. Some mice oscillated between showing no clinical signs and early clinical signs for many months but never progressed to terminal disease. Brain tissue from these mice with chronic subclinical prion disease, sacrificed at >200 days postinoculation, contained high levels of infectivity and showed the presence of PrP^{Sc}. Parallel analysis of brain tissue from mice with terminal disease showed similar levels of infectivity and detectable PrP^{Sc}. These results show that high levels of infectivity and the presence of the abnormal isomer of PrP can be detected in mice with subclinical disease following low-dose prion inoculation.

Prion diseases such as scrapie of sheep, bovine spongiform encephalopathy (BSE) of cattle, and Creutzfeldt-Jakob disease (CJD) of humans are neurodegenerative transmissible diseases. The protein-only hypothesis (21, 35) predicts that the transmissible prion agent consists solely of proteinacious material. During prion disease, PrP^{Sc}, an abnormal isomer of a host protein termed PrP^{c}, accumulates in proteolytic-resistant aggregates. As a consequence, it is proposed that PrP^{Sc} forms part or all of the transmissible prion agent and that it is this abnormal isomer which is responsible for the modification of the structure of PrP^{c}. A feature of prion disease has been the description of distinct prion strains which have been used as evidence for the presence of a replicating genome within the infectious agent (4). The protein-only hypothesis predicts that different prion strains represent different conformations of PrP^{Sc} (38). Different prion inocula may be characterized by several criteria, including their biological properties (33), histopathology (18), and variations in the pattern of PrP^{Sc} deposition (22) and the length of the disease incubation period following experimental inoculation (15).

With respect to prion diseases, the term incubation period is usually defined as the length of time from inoculation to the appearance of clinical signs associated with terminal disease. The incubation period is dependent upon the species and strain of host, the inoculum, and the route of inoculation and is inversely related to the titer of inoculum (34, 36). During the progression of prion disease, infectivity and PrP^{Sc} accumulate in the central nervous system, and in lymphoid tissue following peripheral inoculation, within a clinically silent phase. Following a prion strain-specific incubation period, this preclinical phase usually precipitates into clinically evident terminal disease. However, prion-infected animals that harbor infectivity and appear grossly unaffected during their normal lifetimes following prion inoculation have been reported. Such individuals may be considered to have subclinical as opposed to preclinical infection (12). There are several examples in the literature of chronic subclinical prion infections. Frigg et al. (19) have reported immunodeficient mice with high prion and PrP^{Sc} levels which remain asymptomatic. Race and Chesebro (37) have shown that brain homogenates from mice inoculated with hamster prions harbor infectivity for hamsters, while Hill et al. (23) have demonstrated that hamster prions do indeed replicate in mice without causing clinical disease. A significant feature of all of these reports is that the resultant infectivity in animals with subclinical disease was seen after inoculation with a high dose of inoculum. One intriguing case of subclinical disease has been described by Taylor et al. (39), who have reported intermittent mild or early clinical signs in mice inoculated with low doses of ME7 prion inoculum.

The *Prnp*^{a} mouse-passaged strains ME7 and Rocky Mountain Laboratory (RML) are the two principal mouse inocula which have been used to address which cell types of the immune system interact with or accumulate prions following peripheral prion inoculation (3, 27, 28). These two inocula both originate from scrapied sheep but from different tissues. RML is derived from experimental goats previously inoculated with the sheep scrapie brain homogenate SSBP/1 (8, 11), and ME7 is derived from the spleen of a scrapied sheep (5). Although RML and ME7 have specific incubation periods and induce

* Corresponding author. Mailing address: Centre for Veterinary Science, Department of Clinical Veterinary Medicine, University of Cambridge, Madingley Rd., Cambridge CB3 0ES, United Kingdom. Phone: 44-1223-337655. Fax: 44-1223-337610. E-mail: rb202@cam.ac.uk.

distinct neuropathological characteristics when inoculated into nontransgenic (hereafter referred to as wild-type) mouse strains, they are similar in that their peripheral accumulation is dependent upon the presence of follicular dendritic cells (32). However, it remains to be resolved whether these two prion strains have subtle transmission characteristics that are manifested in differences in affinity for other target cells.

Here we have used the tga20 mouse strain (16) in our initial studies on the comparison of transmission of RML and ME7. This strain of mouse expresses high levels of *Prnp*^{a}-derived PrP and has a relatively short incubation time for prion disease, thereby permitting a rapid disease assessment. We have established that tga20 mice are susceptible to both ME7 and RML inocula, with each producing distinct transmission characteristics. Significantly, we have found that low doses of either ME7 or RML induce subclinical disease in tga20 mice which is associated with high levels of infectivity and the presence of PrP^{Sc}. Our data suggest that events other than merely prion accumulation are responsible for triggering terminal prion disease.

MATERIALS AND METHODS

Mice. The tga20 mouse strain (16) was obtained from Charles Weissmann. Mice were bred in sterile isolators and transferred to conventional animal facilities for inoculation with ME7- or RML-infected brain homogenate. CD-1 Swiss and C57BL/6 wild-type mice were purchased from Harlan UK. CD-1, C57BL/6, and tga20 mice all express the *Prnp*^{a} allelic form of the mouse PrP gene. All regulated procedures involving experimental animals were carried out under project and personal license authority issued in accordance with The Animals (Scientific Procedures) Act 1986.

Genotyping of tga20 mice. The genotype of tga20 mice was verified by PCR using genomic DNA as a substrate. Genomic DNA was isolated by phenol-chloroform extraction from tail snip tissue previously digested in buffer comprising 500 mM Tris HCl (pH 8.0), 20 mM EDTA, 1.0% sodium dodecyl sulfate, 10 mM NaCl, and 150 μg of proteinase K (PK) per ml overnight at 37°C. PCR tubes contained 1.0 U of *Taq* polymerase, 100 ng of substrate DNA, 1× PCR buffer, 200 μM deoxynucleoside triphosphates, 0.1 nM each primer,1.5 mM $MgCl_2$, and 10% dimethyl sulfoxide in a final volume of 25 μl. PCR was carried out in a thermal cycler for 30 cycles after an initial DNA melt of 94°C for 2 min, where one cycle comprised denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. The final extension was for 10 min at 72°C. PCR products were electrophoresed through a 1.5% agarose gel and visualized by ethidium bromide staining and UV transillumination. The primers were as follows: for $PrP^{0/0}$, ATTCGCAGCGCATCGCCTTCTATCGCC; for P10 transgene, GTACCCATAATCAGTGGAACAAGCCCAGC; and for both *Prnp*$^{0/0}$ and transgene, P3-NC (CCCTCCCCCAGCCTAGACCACGA).

Intracerebral inoculations and infectivity bioassay. Mice, either male or female, at 5 to 6 weeks of age were inoculated by intracerebral injection into the right parietal lobe at a depth of 4 to 5 mm. Serial 10-fold dilutions of either ME7 (TSE Resource Centre, Institute of Animal Health, Compton, United Kingdom) or RML brain homogenate were inoculated as a 20-μl volume diluted in phosphate-buffered saline (PBS) or PBS plus 5% bovine serum albumin, respectively. RML 5.0 is derived by passage of RML 4.1 in CD-1 mice. Control tga20 mice were mock injected with PBS or received uninfected CD-1, C57BL/6 brain homogenate, or, subsequently, brain homogenate from these control mice. Initial 10% homogenates were made up in 0.32 M sucrose prior to serial dilution. Inoculated mice were monitored daily for clinical signs of mouse prion disease. The diagnosis of prion disease was based upon that of Dickinson et al. (15). Mice were sacrificed at the point of neurological disease and dysfunction.

Histology and immunocytochemistry. Brain tissue from representative mice for all treatment groups was fixed in buffered formalin for 24 h, inactivated for 1 h with 98% formic acid, soaked in buffered formalin for a further 72 h, and then embedded in paraffin wax. Paraffin sections (5 μm in thickness) were subjected to conventional staining with hematoxylin and eosin. Brain sections from terminally sick mice were examined histologically to confirm scrapie pathology (microvacuolation). Reactive gliosis was confirmed by immunohistochemistry for glial fibrillary acidic protein (GFAP) diluted 1:200 (DAKO, Glostrup, Denmark) and developed with an avidin-biotin labeling kit (Vector Labs, Peterborough, United Kingdom).

Western blot analysis. Brain homogenates were made to 10% (wt/vol) with either homogenate buffer (0.5% Nonidet P-40 and 0.5% sodium deoxycholate in PBS) for RML samples or lysis buffer (0.5% Nonidet P-40, 0.5% sodium deoxycholate, 100 mM NaCl, 10 mM EDTA, and 10 mM Tris HCl [pH 7.4]) for ME7 samples. Samples were treated with PK at a final concentration of 25 μg/ml for 30 min at 37°C. Digestion was terminated by the addition of phenylmethylsulfonyl fluoride. Ten microliters for non-PK-treated samples or 15 μl for PK-treated samples (equivalent to 40 to 50 μg of total protein) was loaded and electrophoresed through a sodium dodecyl sulfate–16% polyacrylamide minigel. Proteins were transferred to nitrocellulose membranes by semidry blotting. Membranes were blocked with TBS-T (10 mM Tris HCl [pH 7.8], 100 mM NaCl, 0.05% Tween 20) plus 5% nonfat milk and subsequently incubated in 1% nonfat milk in TBS-T with XN polyclonal anti-mouse PrP serum (32) (1/1,000) for 1 h, followed by goat anti-rabbit immunoglobulin G–biotin (catalog no. B-7389; Sigma) (1/500) and finally extravidin-horseradish peroxidase (catalog no. E-2886; Sigma) (1/500). All dilutions of antibodies were in 1% nonfat milk in TBS-T. PrP bands were detected by enhanced chemiluminescence.

RESULTS

Identification of tga20 mice. The tga20 mouse line was generated by Fischer et al. (16) by transgenesis of the wild-type PrP gene into Zürich I $PrP^{0/0}$. As a consequence, tga20 mice contain ~50 copies of the PrP transgene and express ~10-fold-higher levels of PrP^c protein. The mice used here were confirmed as being tga20 mice by PCR using primers to detect the wild-type PrP transgene and the Neo-PrP targeting construct used to generate the original $PrP^{0/0}$ line. Genomic DNA from tga20 mice contained the predicted wild-type PrP and Neo-PrP PCR products of 0.8 and 0.7 kb, respectively. Wild-type and $PrP^{0/0}$ mice contain either the 0.8- or 0.7-kb band, respectively (data not shown).

ME7 and RML inocula induce different clinical signs of terminal prion disease. Inoculation of tga20 mice intracerebrally with either ME7- or RML-infected brain homogenate resulted in mouse prion disease with clearly evident, progressive clinical signs, as described in Table 1. Prion disease induced by ME7 or RML normally began with similar early signs. Mice initially displayed dull, ruffled coats and developed hyperactive behavior associated with jerky and uncontrollable running movements. This was soon followed by kyphosis (hunched posture) and a high stepping gait coupled with a tendency to display a straight tail, held parallel to the ground. Head twitching became prominent in both ME7- and RML-infected mice, while those infected with RML were prone to hold their heads to one side (usually the right) when in motion. These early signs of prion disease were followed by neurological indicators of dysfunction. Mice developed proprioceptive deficits as evidenced by clasped feet when raised by the tail. Most mice eventually became extremely lethargic and listless, coupled with having a blank stare. The majority of mice subsequently carried their tails in a C shape. This was followed by mild ataxia which gradually became more severe, and mice progressively displayed a rolling gait. In the late stages of disease, tga20 mice inoculated with RML usually showed more hyperactive behavior than those inoculated with ME7. A significant feature of late terminal disease was that approximately 40% of the RML-infected mice developed pruritus, whereas those inoculated with ME7 showed no signs of scratching. All mice were sacrificed at a standard clinical end point once terminal scrapie symptoms had been established. All mice in individual groups reached terminal disease at very similar times. We compared prion incubation times in males and fe-

TABLE 1. ME7- or RML-induced clinical signs in tga20 mice following intracerebral inoculation

Disease category and clinical signs[a]	% of group showing signs with the following prion inoculum:	
	ME7	RML
Subclinical disease[b]		
Dull, ruffled coat	100	100
Hyperactive	50	100
Kyphosis, high stepping gait	100	100
Straight tail	100	100
Head tilt	10	100
Terminal disease[c]		
Proprioceptive deficits	100	100
Listless, lethargic	100	100
C-shaped tail	60	100
Blank stare	100	100
Progressive ataxia	100	100
Rolling gait	40	100
Pruritus	0	40

[a] Clinical signs used to assess the appearance of ME7- or RML-induced terminal mouse prion disease are shown. Following intracerebral inoculation with prion-infected brain homogenate, mice displayed a variety of clinical signs. These signs are listed in order of appearance from early mild signs to more severe signs associated with terminal disease.

[b] Subclinical disease represents clinical signs that did not always lead to terminal disease. Mice oscillated between having these signs and no signs at all.

[c] Terminal disease represents clinical signs that always progressed to euthanasia.

males to verify whether there was a sex-related difference and to maximize the use of all of the mice from the breeding colony. We found no significant difference in incubation times between males and females following intracerebral inoculation (data not shown). Control tga20 mice were inoculated with brain homogenate from uninfected brains of either C57BL/6 or CD-1 mice, which are the mouse strains used to passage ME7 or RML, respectively. All of the control inoculated tga20 mice remained healthy for the duration of the experiment and did not exhibit clinical signs at any time.

Different incubation times for ME7 and RML in tga20 mice. ME7 and RML inocula injected intracerebrally into tga20 mice induced prion disease with significantly different incubation times (defined here as the time between inoculation and euthanasia at terminal disease), as shown by the data in Table 2. RML prions induced the shortest incubation time for disease onset, and at the highest dose tested this was 58 ± 0.6 days. At this and other high doses of RML, the progression from initial clinical signs to terminal disease was fairly rapid and usually was complete within 3 to 5 days. At lower doses of RML, the incubation time was extended and so was the time taken for mice to display the complete range of clinical signs, which was 12 days at its maximum. The highest concentration of ME7 tested in tga20 mice showed an incubation time of 74 ± 1.1 days for progression to terminal disease, which became more extended with lower doses of inoculum. In contrast to RML, at all of the doses of ME7 which induced terminal disease, progression through the complete range of clinical signs from initial onset to terminal disease proceeded with a fairly fixed time, typically 14 days. The incubation times for different preparations of RML and ME7 in tga20 mice were fairly similar as seen by the regression analysis lines for each inoculum plotted using the least-squares method as described by Prusiner et al. (36) (Fig. 1). A significant difference between ME7 and RML was seen in the infectivity titers of each inoculum calculated using the statistical method of Karber (25). ME7 titers were consistently lower than those of RML, as shown in Table 2. The difference in the incubation period between ME7 and RML may be due to a difference in the titer of these two inocula.

Incubation times of ME7 and RML in nontransgenic wild-type mice. The brain homogenates used to inoculate tga20 mice were derived from terminally sick C57BL/6 or CD-1 mice previously inoculated with ME7 or RML, respectively. The incubation period for ME7- or RML-induced disease in tga20 mice was considerably shorter than that seen in wild-type mice, as shown by the data in Table 3. The incubation period in C57BL/6 mice for high-dose ME7-induced disease was 160 ± 5 days, which is approximately twice as long as that in tga20 mice. Similarly, the incubation period for high-dose RML in CD-1 mice was 134 ± 3 days, which is approximately twofold greater than that seen in tga20 mice. Interestingly, the clinical signs for prion-induced disease and their duration were different for wild-type mice and tga20 mice with respect to each inoculum. C57BL/6 mice inoculated with ME7 showed an extended range of clinical signs compared with tga20 mice. Wild-type mice showed clinical signs for a longer period of time, up to 20 days for ME7 and 28 days for RML, and progressed through each sign more slowly than tga20 mice. In the late stages of disease CD-1 mice with RML appeared to adopt a frozen posture, but unlike C57BL/6 inoculated with ME7, they did not display incontinence.

Subclinical disease in tga20 mice. Mice inoculated with those doses of either ME7- or RML-infected brain homogenate which induced terminal disease showed a clear progression from early to late clinical signs. The onset of ataxia appeared to determine the outcome of the infection. Mice which displayed this particular neurological condition invariably progressed to terminal disease in an irreversible manner. Interest-

TABLE 2. Comparison of ME7 and RML titrations in tga20 mice[a]

Dilution	No. of mice succumbing to terminal disease/no. in group (mean days to terminal disease ± SD) with the following prion inoculum[b]			
	ME7[a]	ME7[b]	RML 4.1	RML 5.0
10^{-1}	5/5 (81 ± 0)	5/5 (74 ± 1)	5/5 (59 ± 3)	5/5 (58 ± 1)
10^{-2}	5/5 (88 ± 2)	5/5 (86 ± 1)	5/5 (62 ± 1)	5/5 (61 ± 1)
10^{-3}	5/5 (110 ± 1)	5/5 (92 ± 3)	5/5 (68 ± 2)	5/5 (67 ± 2)
10^{-4}	5/5 (112 ± 1)	5/5 (96 ± 8)	5/5 (70 ± 1)	5/5 (72 ± 1)
10^{-5}	1/5 (129 ± 0)	5/5 (108 ± 2)	5/5 (73 ± 4)	5/5 (80 ± 1)
10^{-6}	0/5 (>200)	5/5 (142 ± 11)	5/5 (83 ± 1)	5/5 (82 ± 1)
10^{-7}	0/5 (>200)	1/5 (141)	5/5 (85 ± 5)	5/5 (92 ± 3)
10^{-8}	ND[c]	ND	0/5 (>522)	1/5 (89)
10^{-9}	ND	ND	ND	1/5 (100)

[a] tga20 mice were inoculated by the intracerebral route with serial 10-fold dilutions from a 10% (wt/vol) ME7 or RML brain homogenate in a 20-μl volume. Inoculated mice were monitored daily for clinical signs of mouse prion disease. The diagnosis of prion disease was based upon that described in Table 1. Mice were euthanized at the point of neurological disease and dysfunction.

[b] The infectivity titers of the inocula (calculated using the statistical method of Karber [25]) were $10^{6.4}$, $10^{8.4}$, $10^{9.2}$, and $10^{9.6}$ LD_{50}/g of brain for ME7[a], ME7[b], RML 4.1, and RML 5.0, respectively. ME7[a] was acquired from the TSE Resource Centre, IAH, Compton, United Kingdom (supplied by the Neuropathogenesis Unit, Edinburgh, United Kingdom). ME7[b] was prepared at the Centre for Veterinary Science from terminally sick mice previously inoculated with ME7[a].

[c] ND, not done.

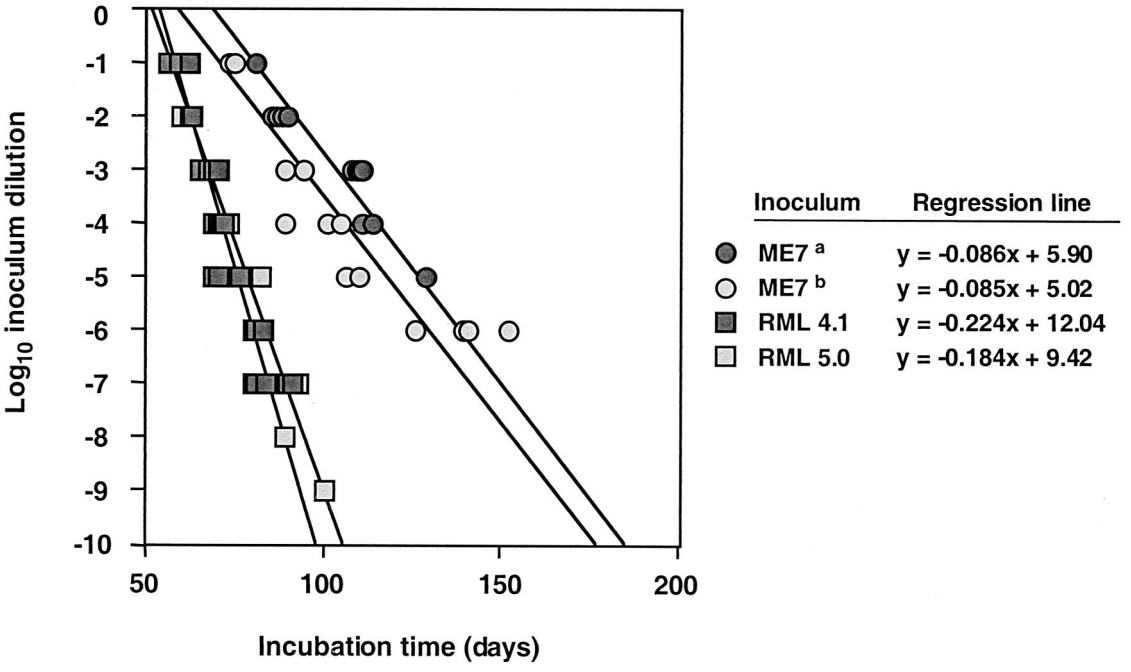

FIG. 1. Regression analysis of ME7 and RML incubation times. The incubation times for different preparations of RML and ME7 in tga20 mice were plotted as a best-fit curve as described by Prusiner et al. (36). Regression analysis equations using the data from Table 2 were calculated using the least-squares method as described by Prusiner et al. (36). ME7[a], ME7 acquired from the TSE Resource Centre, IAH, Compton, United Kingdom (supplied by the Neuropathogenesis Unit, Edinburgh, United Kingdom). ME7[b], ME7 prepared at the Centre for Veterinary Science from terminally sick mice previously inoculated with ME7[a]. For incubation times, $n = 10$; for regression analysis, $n = 5$.

ingly, while mice inoculated with low doses of either ME7 or RML inoculum failed to develop terminal disease, they did show some early clinical signs. Surprisingly, these early signs of disease in tga20 mice did not represent a static situation but rather represented a transient phenomenon. Animals oscillated between normal appearance and behavior and the display of early prion disease but failed to develop ataxia. We reasoned that these mice with subclinical disease may have harbored infectious prions, as seen in other cases of subclinical prion disease (19, 23). To assess this, brain homogenates were prepared from mice with subclinical disease sacrificed at >200 days postinoculation and were subsequently passaged in tga20 mice to determine infectivity titers. The results are shown in Table 4. Intriguingly, brain homogenates from those mice with subclinical prion disease, at least in the case of ME7, appeared to contain as much infectivity as brain homogenates prepared from those animals which had succumbed to terminal prion disease, as shown by the data in Table 4. The appearance of infectivity in brain homogenates from mice with subclinical disease is not simply due to inoculating tga20 mice with brain homogenates. tga20 mice inoculated with uninfected CD-1 or C57BL/6 mouse brain homogenate did not succumb to prion disease, as shown in Table 5. Importantly, brain homogenates from these control mice failed to induce clinical signs of disease following subsequent transmission into additional tga20 mice. This argues against the view that infectivity in mice with subclinical disease represented merely a tga20-to-tga20 transmission phenomenon.

Vacuolation and GFAP staining. Following formic acid inactivation and paraffin embedding, brain sections were stained with hematoxylin-eosin and with antibodies to GFAP (Fig. 2). Figure 2 shows that gliosis (a nonspecific but early indicator of brain damage) was detected in various brain areas surrounding scrapie microcavities. All mice terminally sick with scrapie displayed vacuolar changes and strong reactive gliosis with a distinct regional profile that was specific to ME7 or RML. Interestingly, comparing RML and ME7, which originate from scrapied sheep brain (4, 11) and spleen (5), respectively, we observed different pathologies in transgenic mice overexpressing mouse PrP but not in wild-type mice. It was surprising to see no difference in the pattern of pathology between ME7 and RML in wild-type mice. RML is regarded as similar to the mouse prion strain 139A. Both are derived from the Chandler

TABLE 3. Mean incubation times for ME7- or RML-induced mouse scrapie following intracerebral inoculation into tga20, CD-1, or C57BL/6 mice[a]

Mouse strain	Incubation time (days, mean ± SD) with the following prion inoculum:	
	ME7[a]	RML 4.1
tga20	78 ± 2	59 ± 3
CD-1	ND[b]	134 ± 3
C57BL/6	160 ± 5	145 ± 7

[a] Female tga20 or nontransgenic wild-type mice ($n = 10$) were inoculated by the intracerebral route with 20 μl of a 10^{-1} dilution of a 10% (wt/vol) ME7 or RML brain homogenate. Inoculated mice were monitored daily for clinical signs of mouse prion disease. The diagnosis of prion disease was according to that described in Table 1. Mice were euthanized at the point of neurological disease and dysfunction. ME7[a], ME7 acquired from the TSE Resource Centre, IAH, Compton, United Kingdom (supplied by the Neuropathogenesis Unit, Edinburgh, United Kingdom).

[b] ND, not done.

TABLE 4. Prion infectivity in the central nervous systems of mice with subclinical ME7- or RML-induced disease[a]

Inoculum[b]	Dilution[c]	No. of mice succumbing to terminal disease/no. of mice in group	Mean days to terminal disease ± SD	Titer (LD_{50}/g of brain)[d]
ME7[a]	10^{-4} (terminal)	5/5	84 ± 4	$10^{5.1}$
		5/5	83 ± 4	
	10^{-5} (terminal)	5/5	79 ± 0	$10^{5.5}$
		5/5	79 ± 1	
	10^{-6} (subclinical)	5/5	87 ± 1	$10^{4.8}$
		5/5	87 ± 1	
	10^{-7} (subclinical)	5/5	82 ± 4	$10^{4.7}$
		5/5	95 ± 2	
RML 5.0	10^{-6} (terminal)	5/5	73 ± 3	$10^{5.1}$
		5/5	78 ± 4	
	10^{-7} (terminal)	5/5	82 ± 0	$10^{4.9}$
		5/5	72 ± 1	
	10^{-8} (subclinical)	5/5	88 ± 1	$10^{2.9}$
		5/5	87 ± 1	
	10^{-9} (subclinical)	4/5	130 ± 4	>1
		3/5	127 ± 0	

[a] Twenty-microliter volumes of 10% (wt/vol) brain homogenates prepared from tga20 mice that had succumbed to terminal prion disease or from mice with subclinical disease that were euthanized at >200 days after prion inoculation were inoculated by the intracerebral route into tga20 mice. Inoculated mice were monitored daily for clinical signs of mouse prion disease. The diagnosis of prion disease was based upon that described in Table 1. Mice were euthanized at the point of neurological disease and dysfunction.

[b] ME7[a], ME7 acquired from the TSE Resource Centre, IAH, Compton, United Kingdom (supplied by the Neuropathogenesis Unit, Edinburgh, United Kingdom).

[c] The dilutions listed represent those inoculated into the primary mice according to Table 2.

[d] Prion titers were calculated according to the formulae described in Figure 1.

strain, and profound differences between ME7 and 139A lesion profiles in wild-type mice are reported (17).

Detection of PrP^{Sc} in animals with subclinical disease. PrP^{Sc} was detected in brain stems from tga20 mice that had succumbed to terminal prion disease and was also seen in samples from mice exhibiting subclinical disease, as shown in Fig. 3. ME7 and RML brain stem homogenates from tga20 mice with subclinical disease showed different susceptibilities to PK digestion, and each could be satisfactorily digested only in separate buffers. RML PrP^{Sc} was most sensitive to PK treatment in homogenate buffer, whereas ME7 was digested only in lysis buffer. This differential susceptibility to PK digestion in different buffers was maintained for brain homogenates from wild-type mice that had succumbed to terminal prion disease, and like tga20 mice, all showed the presence of PrP^{Sc}. Although it was not formally quantified, more PrP^{Sc} appeared to be present in samples of ME7-inoculated brain tissue than in RML-inoculated samples.

DISCUSSION

The agent responsible for prion disease may exist in different forms, each carrying the specific information which determines its own distinct biological properties, such as incubation period, lesion profile, and PrP^{Sc} glycoform pattern. The protein-only hypothesis predicts that variation between different prion inocula, including those from the same species, is enciphered in the different conformations which may be taken up by PrP^{Sc}. Here we have compared the transmission characteristics of the mouse prion inocula ME7 and RML in tga20 mice. Both of these inocula are derived from natural sheep scrapie material, although each originates from a different tissue site. RML is from the scrapied brain homogenate SSBP/1 after transmission to goats and is passaged in CD-1 mice (8). In contrast, the ME7 inoculum was isolated by passage of scrapied sheep spleen tissue in RIII mice (a subline of the C57BL/6 strain) (13, 14, 40).

Different transmission characteristics are evident for ME7 and RML in tga20 mice. RML induced the most rapid onset of terminal disease, which was reached after only 58 days of incubation following inoculation with a high dose of inoculum injected by the intracerebral route. In contrast, the highest dose of ME7 induced terminal disease after 74 days. These incubation times are considerably shorter, by approximately 50%, than those seen in the wild-type mice used to routinely passage these inocula. The differences in incubation times between ME7 and RML in tga20 mice was maintained between different preparations of each inoculum as seen by the comparison of regression analysis lines. On each occasion tested, the RML inoculum showed a higher titer than ME7, and our titer values are consistent with those seen by others. The RML inoculum routinely displays a titer of $\sim 10^{9.5}$ 50% lethal doses (LD_{50})/g of brain in tga20 mice (M. A. Klein and A. Aguzzi, unpublished data), while ME7 has a titer of $\sim 10^{8}$ (39) in C57BL/6 mice. Collectively, our transmission studies show that tga20 mice can successfully be used to assess both ME7 and RML infectivities and that these different inocula induce terminal disease with a distinct set of clinical signs. Histological examination of brain sections from prion-inoculated tga20 mice with terminal disease revealed that ME7 caused a more general and extensive pattern of vacuolation than RML.

In experimentally inoculated mice, the onset of terminal disease is usually signaled by the appearance of early mild clinical signs, such as head shaking or excitability, which progress to late-stage signs, such as proprioceptive defects and severe ataxia. All of the mice inoculated in this study which developed ataxia invariably succumbed to terminal prion disease. However, careful observation of inoculated mice identified those which developed intermittent mild signs of disease but did not progress to ataxia. These mice had received a low dose of inoculum. Intermittent or reversible clinical signs in

TABLE 5. Control brain homogenates inoculated by the intracerebral route into tga20 mice[a]

Homogenate	No. of mice succumbing to terminal disease/no. of mice in group	Incubation time (days postinoculation)
tga20[b]	0/5	>309
C57BL/6	0/5	>612
CD-1	0/5	>612
tga20-passaged C57BL/6[c]	0/5	>295
tga20-passaged CD-1	0/5	>295

[a] Brain homogenates (20 μl per mouse, 10% [wt/vol]) prepared from uninfected tga20, C57BL/6, or CD-1 mice were inoculated by the intracerebral route into tga20 mice. The tga20 mice previously inoculated with uninfected C57BL/6 or CD-1 brain homogenates were sacrificed at >300 days post inoculation, and 10% (wt/vol) brain homogenates were passaged into further tga20 recipients. All mice were monitored daily and did not show any clinical signs.

[b] Original brain material used to inoculate tga20 recipients.

[c] Second-passage brain material.

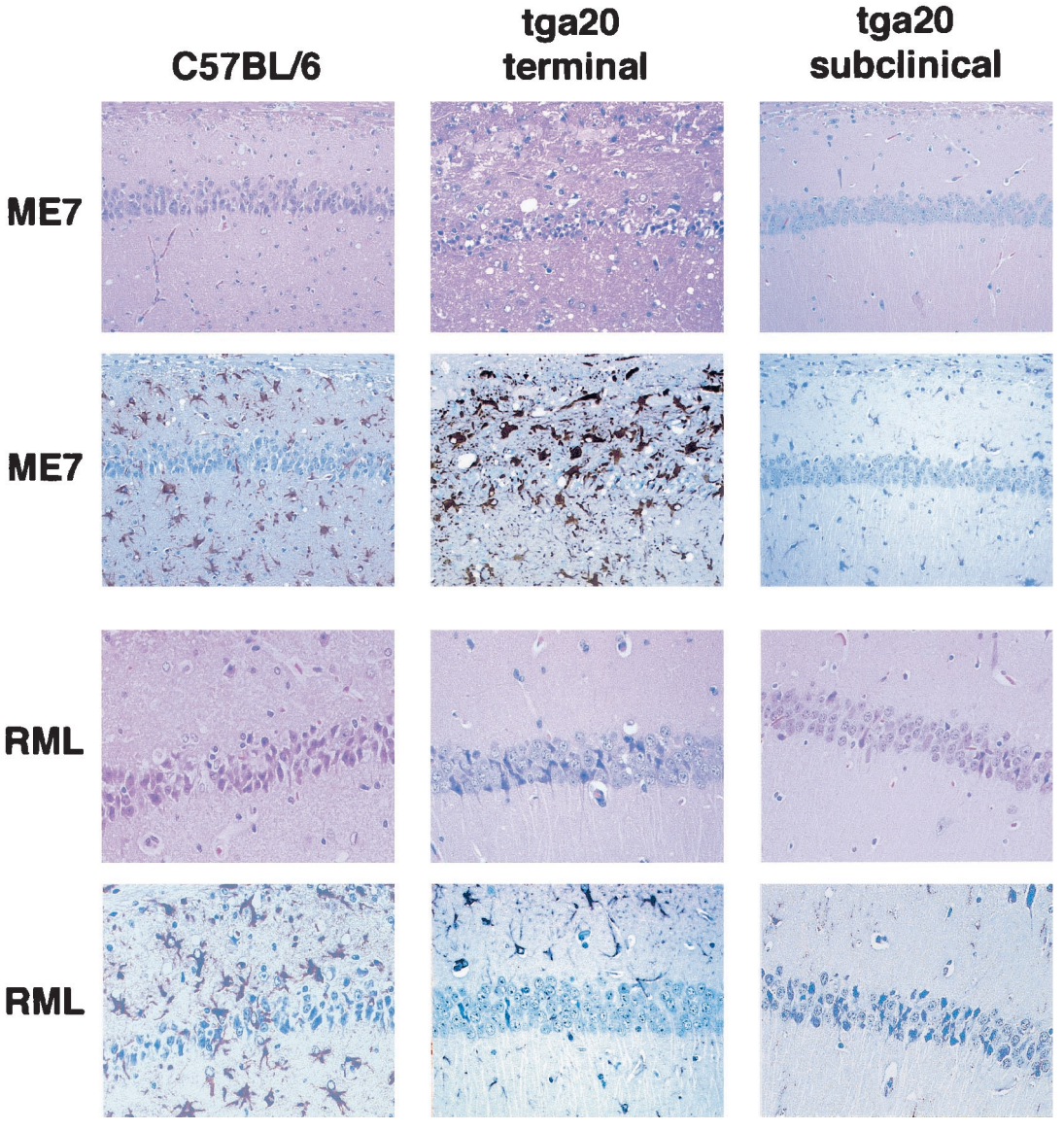

FIG. 2. Histology and immunocytochemistry. Sections of brain tissue were subjected to conventional staining with hematoxylin and eosin (rows 1 and 3) and examined histologically to confirm prion pathology by the presence of microvacuolation. Reactive gliosis was confirmed by immunohistochemistry for glial fibrillary acidic protein (rows 2 and 4). Magnification, ×250.

wild-type mice inoculated with low doses of ME7 have been described before (39). Here we report a similar finding following inoculation of tga20 mice with an ME7 or RML prion inoculum. However, in contrast to the study by Taylor et al. (39), where the oscillating clinical signs culminated in terminal disease, in the experiments reported here, the oscillating clinical signs have not reached terminal disease. Transmission of brain homogenates prepared from ME7- or RML-inoculated tga20 mice with subclinical disease induced terminal disease in additional tga20 mice and with incubation times similar to those for brain homogenates from mice with terminal disease. The level of infectivity in subclinical ME7 brain homogenates was $\sim 10^5$ LD_{50}/g of brain in both groups of mice tested, and this value was similar to that from mice that succumbed to terminal disease. All of these prion titers were significantly greater than the amount inoculated. A similar trend was seen following RML inoculation, although the amount of infectivity associated with subclinical brain homogenates titrated out in the dilutions tested. The failure of uninfected tga20 brain homogenate to cause any disease after inoculation into additional tga20 mice implies that the infectivity in animals with subclinical disease does represent authentic prion accumulation. There are further examples in the literature of chronic subclinical infections with prions. Mice heterozygous for PrP gene disruption show high prion and PrP^{Sc} levels but a delayed onset of disease (7), immunodeficient mice do not succumb to disease despite high levels of prions and PrP^{Sc} (19), and hamster prions can persist (37) and replicate (23) in mice in a clinically silent fashion. In all of these studies infectivity in animals with subclinical disease was seen after inoculation with relatively high doses of inoculum. The intriguing feature of the subclinical disease infectivity reported here is that it was in-

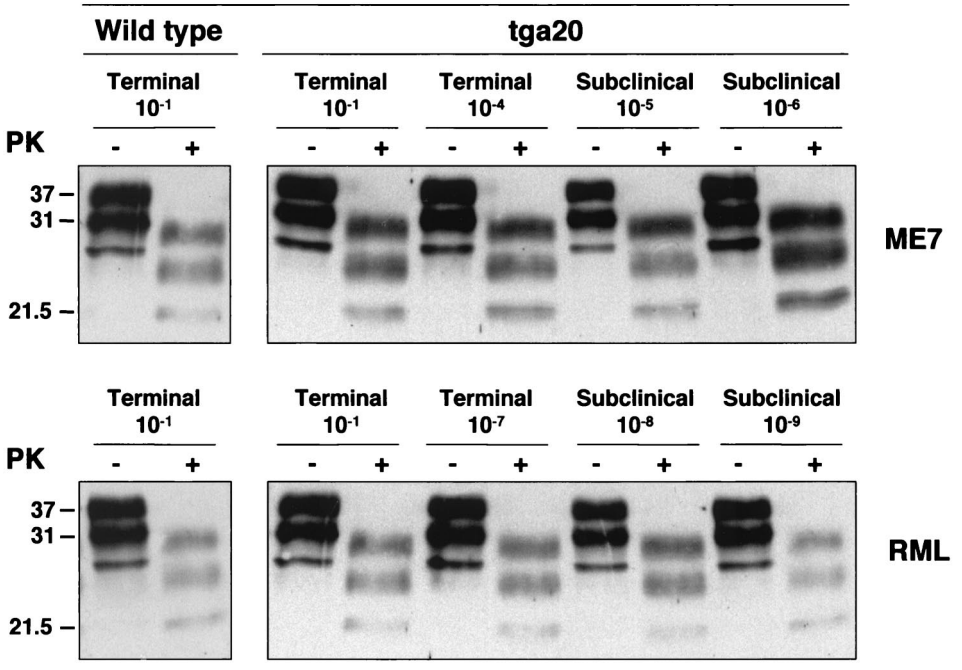

FIG. 3. Western blot analysis of PrP^{Sc} in brain stem homogenates from ME7- or RML-infected mice with terminal and subclinical disease. Aliquots of 10% (wt/vol) brain stem homogenates prepared from ME7- or RML-inoculated mice were treated with PK, followed by Western blotting with XN polyclonal anti-mouse PrP serum and enhanced chemiluminescence. Ten microliters for non-PK-treated samples and 15 μl for PK-treated samples (equivalent to 40 to 50 μg of total protein) were loaded. The left-hand panels show the positive control sample from wild-type mice inoculated with a 10^{-1} dilution. ME7 (TSE Resource Centre) and RML 5.0 terminal and subclinical brain stem homogenates are shown. Numbers on the left are molecular masses in kilodaltons.

duced by dilutions of inoculum that were below the end point of titration.

A second feature of our findings was the detection of PrP^{Sc} in the brains of tga20 mice with subclinical prion disease. When we assessed the levels of PrP^{Sc} in whole brain homogenates from the tga20 mice, we could detect only small amounts in the group with subclinical disease inoculated with RML at a dilution of 10^{-9} and in both groups with subclinical disease inoculated with ME7 (data not shown). We were routinely unable to detect significant levels of PrP^{Sc} in whole brain homogenates from the terminally sick mice for both prion strains. However, when we tested brain stem samples only, we were able to detect PrP^{Sc} in all groups of mice, as shown in Fig. 3. Unlike in tga20 mice, PrP^{Sc} was detectable in the whole brain homogenates of wild-type animals, which express normal levels of PrP^{c}. Other reports have shown that while tga20 mice can succumb to terminal disease after a very short incubation time, PrP^{Sc} in whole brain homogenates was present at low levels (16); in our case it was undetectable when assessed by Western blotting. Several possibilities exist for this apparent lack of, or small amounts of, PrP^{Sc} in tga20 mice with terminal disease. First, disease may occur rapidly in tga20 mice prior to appreciable accumulation of PrP^{Sc} in regions of the brain other than the brain stem. Second, the resultant PrP^{Sc} may be highly sensitive to PK digestion, giving the impression that there is little, if any, of this form of PrP, as reported for other examples of prion disease (9, 24, 29, 30). In this respect, it is interesting that brain homogenates from tga20 mice inoculated via the sciatic nerve (20), which were associated with terminal disease incubation times of up to 90 days, did not show much if any PrP^{Sc}. It is possible that the higher levels of PrP^{Sc} which accumulate in the tga20 mice with subclinical disease do so as a consequence of the extended duration of the disease process. A third reason for lack of appreciable PrP^{Sc} formation in tga20 mice when whole brain homogenates were assessed is that the transgenic nature of PrP in these mice may render this material inherently unstable and/or readily metabolized. Although ME7 and RML required different buffers for efficient PK cleavage to detect PrP^{Sc}, the same buffer was effective for brain tissue from both tga20 mice with subclinical disease and wild-type mice with terminal disease. This suggests that ME7 and RML PrP^{Sc} retains some conformational similarity, and therefore stability, when expressed in the different mouse strains.

The accumulation of PrP^{Sc} is the only established by-product of prion disease, and its detection is routinely used for biochemical diagnosis of transmissible spongiform encephalopathies (26). Molecular analysis of PrP^{Sc} through quantitative assessment of the ratios of the different glycoforms and molecular mass measurement of unglycosylated proteins have been used to distinguish different prion inocula (2, 6, 10). However, while the PK and Western blot procedure is suggested to be of comparable sensitivity to heterologous bioassay for BSE or CJD material in mice, the test is essentially nonquantitative and difficult to standardize. A critical feature of the assay is the concentration of PK used. If the concentration of PK used is too high, the enzyme may remove small quantities of PrP^{Sc}. Conversely, too low a concentration of enzyme may not remove all of the relatively large amount of PrP^{c}. Other markers of prion disease are required. Barnard et al. (1) have described a sensitive time-fluorescence immunoassay

which distinguishes normal cattle from BSE-infected cattle by measurement of insoluble PrP, which increases during disease. Miele et al. (31) have reported that erythroid differentiation-related factor (EDRF) is decreased in spleen tissue and bone marrow of ME7-inoculated mice with terminal disease. Significantly, levels of EDRF mRNA were decreased well in advance of any clinical signs. Analysis of either insoluble PrP or EDRF RNA may aid the diagnosis of subclinical prion disease.

Collectively, our observations with tga20 mice suggest that events other than merely prion infectivity and PrP^{Sc} accumulation are responsible for triggering terminal prion disease. Our data also suggest that susceptibility to prion disease should not be judged solely by the development of terminal clinical signs. It is presently unknown if subclinical prion disease exists within individuals, either human or animal, with natural transmissible spongiform encephalopathies, and this is an important point to elucidate. Our data, here using same-species transmission, show that high levels of infectivity can accumulate within infected mice following exposure to extremely low doses of inoculum. It will be important to carry out similar low-dose prion transmission studies using xenogeneic inocula to assess the risks associated with cross-species prion infectivity. These data may have important implications for public health regarding iatrogenic transmission of variant CJD from apparently healthy individuals previously exposed to BSE-contaminated bovine products.

ACKNOWLEDGMENTS

This work was supported by a BBSRC research grant.
We are grateful to Charles Weissmann for providing the tga20 mice.

REFERENCES

1. **Barnard, G., B. Helmick, S. Madden, C. Gilbourne, and L. R. Pate.** 2000. The measurement of prion protein in bovine brain tissue using differential extraction and DELFIA as a diagnostic test for BSE. Luminescence **15:**357–362.
2. **Baron, T. G. M., J.-Y. Madec, D. Calavas, Y. Richard, and F. Barillet.** 2000. Comparison of French natural scrapie isolates with bovine spongiform encephalopathy and experimental scrapie infected sheep. Neurosci. Lett. **284:** 175–178.
3. **Brown, K. L., K. Stewart, D. L. Ritchie, N. A. Mabbott, A. Williams, H. Fraser, W. I. Morrison, and M. E. Bruce.** 1999. Scrapie replication in lymphoid tissues depends on prion protein-expressing follicular dendritic cells. Nat. Med. **5:**1308–1312.
4. **Bruce, M. E., and A. G. Dickinson.** 1979. Biological stability of different classes of scrapie agent, p. 71–86. *In* S. B. Prusiner and W. J. Hadlow (ed.), Slow transmissible diseases of the nervous system, vol. 2. Academic Press, New York, N.Y.
5. **Bruce, M. E., A. G. Dickinson, and H. Fraser.** 1976. Cerebral amyloidosis in scrapie in the mouse: effect of agent strain and mouse genotype. Neuropathol. Appl. Neurobiol. **2:**471–478.
6. **Bruce, M. E., R. G. Will, J. W. Ironside, I. McConnell, D. Drummond, A. Suttie, L. McCardle, A. Chree, J. Hope, C. Birkett, S. Cousens, H. Fraser, and C. J. Bostock.** 1997. Transmissions to mice indicate that 'new variant' CJD is caused by the BSE agent. Nature **389:**498–501.
7. **Bueler, H., A. Raeber, A. Sailer, M. Fischer, A. Aguzzi, and C. Weissmann.** 1994. High prion and PrPSc levels but delayed onset of disease in scrapie-inoculated mice heterozygous for a disrupted PrP gene. Mol. Med. **1:**19–30.
8. **Chandler, R. L.** 1961. Encephalopathy in mice produced by inoculation with scrapie brain material. Lancet **i:**1378–1379.
9. **Collinge, J., M. S. Palmer, J. Sidle, R. Gowland, J. Medori, J. Ironside, and P. Lnatos.** 1995. Transmission of fatal insomnia to laboratory animals. Lancet **346:**569–570.
10. **Collinge, J., K. C. Sidle, J. Meads, J. Ironside, and A. F. Hill.** 1996. Molecular analysis of prion strain variation and the aetiology of 'new variant' CJD. Nature **383:**685–690.
11. **Dickinson, A. G.** 1976. Scrapie in sheep and goats, p. 209–241. *In* R. H. Kimberlin (ed.), Slow virus diseases of animals and man. Elsevier/North-Holland, Amsterdam, The Netherlands.
12. **Dickinson, A. G., H. Fraser, and G. W. Outram.** 1975. Scrapie incubation time can exceed natural lifespan. Nature **256:**732–733.
13. **Dickinson, A. G., and V. M. Meikle.** 1969. A comparison of some biological characteristics of the mouse-passaged scrapie agents, 22a and ME7. Genet. Res. **13:**213–225.
14. **Dickinson, A. G., V. M. Meikle, and H. Fraser.** 1969. Genetic control of the concentration of ME7 scrapie agent in the brain of mice. J. Comp. Pathol. **79:**15–22.
15. **Dickinson, A. G., V. M. Meikle, and H. Fraser.** 1968. Identification of a gene which controls the incubation period of some strains of scrapie agent in mice. J. Comp. Pathol. **78:**293–299.
16. **Fischer, M., T. Rulicke, A. Raeber, A. Sailer, M. Moser, B. Oesch, S. Brandner, A. Aguzzi, and C. Weissmann.** 1996. Prion protein (PrP) with amino-proximal deletions restoring susceptibility of PrP knockout mice to scrapie. EMBO J. **15:**1255–1264.
17. **Fraser, H.** 1979. Neuropathology of scrapie: the precision of the lesions and their diversity, p. 307–406. *In* S. B. Prusiner and W. J. Hadlow (ed.), Slow transmissible diseases of the nervous system, vol. 1. Academic Press, New York, N.Y.
18. **Fraser, H., and A. G. Dickinson.** 1968. The sequential development of the brain lesion of scrapie in three strains of mice. J. Comp. Pathol. **78:**301–311.
19. **Frigg, R., M. A. Klein, I. Hegyi, R. M. Zinkernagel, and A. Aguzzi.** 1999. Scrapie pathogenesis in subclinically infected B-cell-deficient mice. J. Virol. **73:**9584–9588.
20. **Glatzel, M., and A. Aguzzi.** 2000. PrP^{C} expression in the peripheral nervous system is a determinant of prion neuroinvasion. J. Gen. Virol. **81:**2813–2821.
21. **Griffith, J. S.** 1967. Self-replication and scrapie. Nature **215:**1043–1044.
22. **Hecker, R., A. Taraboulos, M. Scott, K. M. Pan, S. L. Yang, M. Torchia, K. Jendroska, S. J. DeArmond, and S. B. Prusiner.** 1992. Replication of distinct scrapie prion isolates is region specific in brains of transgenic mice and hamsters. Genes Dev. **6:**1213–1228.
23. **Hill, A. F., S. Joiner, J. Linehan, M. Desbruslais, P. L. Lantos, and J. Collinge.** 2000. Species-barrier-independent prion replication in apparently resistant species. Proc. Natl. Acad. Sci. USA **97:**10248–10253.
24. **Hsiao, K. K., M. Scott, D. Foster, D. F. Groth, S. J. DeArmond, and S. B. Prusiner.** 1990. Spontaneous neurodegeneration in transgenic mice with mutant prion protein. Science **250:**1587–1590.
25. **Karber, G.** 1931. Beitrag zur kollecetiven Behandlung Pharmakologische Reihen versuche. Arch. Exp. Pathol. Pharmacol. **162:**480–483.
26. **Kascsak, R. J., R. Fersko, D. Pulgiano, R. Rubenstein, and R. I. Carp.** 1997. Immunodiagnosis of prion disease. Immunol. Investig. **26:**259–268.
27. **Klein, M. A., R. Frigg, E. Flechsig, A. J. Raeber, U. Kalinke, H. Bluethmann, F. Bootz, M. Suter, R. M. Zinkernagel, and A. Aguzzi.** 1997. A crucial role for B cells in neuroinvasive scrapie. Nature **390:**687–690.
28. **Klein, M. A., R. Frigg, A. J. Raeber, E. Flechsig, I. Hegyi, R. M. Zinkernagel, C. Weissmann, and A. Aguzzi.** 1998. PrP expression in B lymphocytes is not required for prion neuroinvasion. Nat. Med. **4:**1429–1433.
29. **Lasmezas, C. I., J. P. Deslys, O. Robain, A. Jaegly, V. Beringue, J. M. Peyrin, J. G. Fournier, J. J. Hauw, J. Rossier, and D. Dormont.** 1997. Transmission of the BSE agent to mice in the absence of detectable abnormal prion protein. Science **275:**402–405.
30. **Medori, R., P. Montagna, H. J. Tritschler, A. LeBlanc, P. Cortelli, P. Tinuper, E. Lugaresi, and P. Gambetti.** 1992. Fatal familial insomnia: a second kindred with mutation of prion protein gene at codon 178. Neurology **42:** 669–670.
31. **Miele, G., J. Manson, and M. Clinton.** 2001. A novel erythroid-specific marker of transmissible spongiform encephalopathies. Nat. Med. **7:**361–364.
32. **Montrasio, F., R. Frigg, M. Glatzel, M. A. Klein, F. Mackay, A. Aguzzi, and C. Weissmann.** 2000. Impaired prion replication in spleens of mice lacking functional follicular dendritic cells. Science **288:**1257–1259.
33. **Pattison, I. H., and G. C. Millson.** 1961. Scrapie produced experimentally in goats with special reference to the clinical syndrome. J. Comp. Pathol. **71:**101–108.
34. **Pattison, I. H., and K. Smith.** 1963. Histological observations on experimental scrapie in the mouse. Res. Vet. Sci. **4:**269–275.
35. **Prusiner, S. B., D. C. Bolton, D. F. Groth, K. A. Bowman, S. P. Cochran, and M. P. McKinley.** 1982. Further purification and characterization of scrapie prions. Biochemistry **21:**6942–6950.
36. **Prusiner, S. B., S. P. Cochran, D. F. Groth, D. E. Downey, K. A. Bowman, and H. M. Martinez.** 1981. Measurement of the scrapie agent using an incubation time interval assay. Ann. Neurol. **11:**353–358.
37. **Race, R., and B. Chesebro.** 1998. Scrapie infectivity found in resistant species. Nature **392:**770.
38. **Safar, J., H. Wille, V. Itri, D. Groth, H. Serban, M. Torchia, F. E. Cohen, and S. B. Prusiner.** 1998. Eight prion strains have PrP^{Sc} molecules with different conformations. Nat. Med. **4:**1157–1165.
39. **Taylor, D. M., I. McConnell, and C. E. Ferguson.** 2000. Closely similar values obtained when the ME7 strain of scrapie agent was titrated in parallel by two individuals in separate laboratories using two sublines of C57BL mice. J. Virol. Methods **86:**35–40.
40. **Zlotnik, I., and J. C. Rennie.** 1965. Experimental transmission of mouse passaged scrapie to goats, sheep, rats and hamsters. J. Comp. Pathol. **75:** 147–157.