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Characteristics of scrapie isolates derived from hay mites

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Previous epidemiological evidence suggested that in some instances a vector and/or reservoir is involved in the occurrence and spread of transmissible spongiform encephalopathies (TSEs). In a preliminary study, hay mite preparations from five Icelandic farms with a history of scrapie were injected into mice, and some of these mice became sick after long incubation periods. To confirm that the disease was scrapie, subsequent passages in mice were performed. In addition, the characteristics of the disease process in these passages were assessed and the results compared to those findings with standard scrapie strains. As expected for scrapie, subsequent passages in the same host led to shortened incubation periods compared to those in primary isolate mice, and all mice had spongiform changes in brain. Results were similar for three of four isolates with regard to clinical manifestations, the incubation periods in mice of the three scrapie incubation-period genotypes (s7s7, s7p7, p7p7), and the PrP^{Sc} Western blot (WB) pattern. The characteristics of the fourth isolate were markedly different from the other three isolates with regard to these parameters. Comparison of the characteristics of standard mouse-adapted scrapie strains and the four isolates revealed differences; these differences were particularly pronounced for the fourth isolate. *Journal of NeuroVirology* (2000) **6,** 137–144.

Keywords: scrapie; hay mites; primary isolates; genetic markers; incubation period

Introduction

A number of findings in the epidemiology of the transmissible spongiform encephalopathies (TSEs) (also known as prion diseases or unconventional slow infections) suggest involvement of a vector and/or reservoir (Palsson, 1979; Brown *et al*, 1987). For the human group of diseases, sporadic cases of Creutzfeldt-Jakob disease (CJD) occur in the absence of any demonstrable genetic predisposition or of any contact with other cases (Brown *et al*, 1987). With scrapie, the archetypical TSE, which is a natural disease in sheep and goats, the disease can appear suddenly in a flock in the absence of any known exposure to infected flocks (Palsson, 1979). Finally, fields in Iceland, that were left empty for up to 3 years after the destruction of scrapie-infected flocks, were repopulated with known scrapie-free sheep, and some of the sheep in this latter group subsequently developed scrapie (Palsson, 1979). This last 'experiment in nature' has yielded similar results a number of times in Iceland and in the United Kingdom. In one Icelandic farm, flocks have been eradicated three times; each time, the farm was left without sheep for 2 years, and after restocking with sheep from scrapie-free areas, the disease reappeared. Several years ago, a suggestion was made (S Sigurdarson, personal communication) that hay mites would be a good candidate as a vector for scrapie; this led to infection of mice with mite samples prepared from hay obtained from five Icelandic farms. Ten of these 71 mice became sick after injection with mite preparations from three of the five farms (Wisniewski *et al*, 1996; Rubenstein *et al*, 1998). The incubation periods ranged from 340 days to 626 days, and these mice had the protease-resistant

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form, Prp^{Sc}, of a host-coded glycoprotein, PrP^{c}. The protease-resistant form is a marker of TSE disease (Prusiner, 1991; Parchi *et al*, 1996). For some of these clinically positive mice, the banding pattern on WB analysis was unique (Wisniewski *et al*, 1996; Rubenstein *et al*, 1998).

A number of factors can confound the interpretation of positive findings during primary isolations of scrapie. First, if the incubation period extends into old age, aging effects can be confused with clinical manifestations of scrapie. Second, concurrent disease can lead to difficulty in interpretation of sick mice that are seen toward the end of life. For these reasons, it is imperative that primary isolates are confirmed by a subsequent passage into the same host species (Fraser, 1983). Based on previous findings for passages after crossing the species barrier, incubation periods on subsequent passages should be shorter than that obtained on primary isolation (Fraser, 1983; Kimberlin, 1979). If subsequent passages of primary isolates are successful, characterization of the biological, histopathological and PrP^{Sc} parameters of the isolates becomes important. Comparison of the characteristics of the isolates to the well-defined characteristics of the scrapie strains used in previous studies may provide clues concerning the origin of the isolates: if the characteristics of mite isolates are different from those of any scrapie strains used in the laboratory, it would indicate a mite origin for the isolates and virtually rule out laboratory contamination, whereas if the characteristics are the same as those of a laboratory strain, the isolates may or may not be from the original source. In the current study, we did first and second passages of a number of primary isolates, and the characteristics of several of the isolates were examined in detail.

Results

The clinical manifestations of scrapie can differ markedly as a function of the strain of agent. For example, in goats, the disease can manifest itself as either a drowsy or a scratchy form (Dickinson and Fraser, 1979). Also, in hamsters, a key finding with the commonly used 263K strain is head-bobbing. In contrast, for a number of other hamster-adapted strains (e.g., 139H, 22CH, ME7H), there is increasing lethargy and mild incoordination, but head bobbing is never observed (Carp *et al*, 1990). In the current studies, homogenates prepared from mice that were positive on primary isolation were injected i.c. into mice; this was termed 'first passage.' Isolates 1, 2 and 3 (Table 1) yielded mice with the common clinical features of scrapie in mice: incoordination, failure to walk efficiently on a grid (Carp *et al*, 1984), weakness, paresis and paralysis and eventual death. In contrast, isolate 4 caused marked obesity, progressive lethargy and somnolence followed by sudden death, with the mice rarely showing the motor dysfunction that is characteristic of the standard clinical features of scrapie in mice.

Table 1 Incubation periods for primary isolate and passage 1 (mean ± s.e.) of mite preparations

	Primary isolate		*Passage 1*[(*)]		
Isolate No.	*Route of injection*	*CW mice*	*s7s7 CW*	*s7p7 F1*	*p7p7 IM*
1	i.p.	340[(a)]	~158[(b)]	244 ± 2	278 ± 3
2	i.c.	376	~158[(b)]	233 ± 3	288 ± 6
3	i.p.	376	~158[(b)]	239 ± 3	311 ± 4
4	i.c.	501	293 ± 13	439 ± 0	515[(c)]

[(*)]All second-passage mice were injected i.c.; [(a)]Day post-injection when mouse was sacrificed; [(b)]Mice were all clinicallly positive when first scored at 158 days; [(c)]Mice appeared clinically normal when killed, but contained PrP^{Sc}.

Scrapie incubation periods in various inbred mice is the marker used most frequently to delineate mouse-adapted strains of scrapie. Inbred mice have a gene, Sinc (Dickinson and Meikle, 1969) (also termed Prn-i) (Carlson *et al*, 1986), which is a major controlling influence on incubation periods of scrapie strains. The ME7 strain and most other scrapie strains have a short incubation period in s7s7 mice and a long incubation period in mice with the p7p7 allele. In contrast, strains such as 22A have a longer incubation period in s7s7 than in p7p7 mice. The incubation period in s7p7 mice can be quite informative; for example, for some ME7-like strains, the incubation period falls between those of the homozygotes, whereas for other strains, the incubation period is longer than in either homozygote. As shown in Table 1, the incubation periods of isolates 1, 2 and 3 were similar in mice with the s7s7 Sinc genotype (CW); furthermore, the incubation periods of the three isolates were similar to each other in s7p7 (F1) as well as in p7p7 mice (IM). The incubation period of isolate 4 in each of the mouse Sinc genotypes was much longer than those of isolates 1, 2 or 3. For both groups, the incubation period was shortest in s7s7 mice and longest in p7p7 mice.

The PrP^{Sc} pattern in WB of isolates 1, 2 and 3 (Figure 1, lanes 1, 2 and 3) yielded a pattern similar to those of several scrapie strains, e.g., ME7 (Figure 1, lane 5) and 22L (data not shown). The patterns for these three isolates were the same upon primary isolation as well as in first- and second-passage mice, and the strain of mouse did not affect the pattern (Figure 2). The findings for isolate 4 were different in the brains of primary isolate and first-passage CW mice. In preparations of these brains, there were two bands, one slightly higher (>30 kDa) than the highest of the standard bands and one slightly lower (<20 kDa) than the lowest band (Figure 1, lane 4). The preparation in lane 4 of

Figure 1 is from a primary isolate CW mouse. A similar pattern was seen on the first mouse-to-mouse passage in CW mice, whereas first passage in IM and F1 mice (s7p7) and second passage in CW, SJL and C57BL mice yielded three bands.

The data from Table 1 and Figure 1 were combined with results from subsequent passages and are presented in the flow charts shown in Figures 2 and 3. From the group of three isolates with similar characteristics, the flow chart for isolate 2 is shown in Figure 2. In addition to the results from the initial experiment (data from Table 1), incubation periods from a subsequent injection of brain homogenates from primary isolate mice into two s7s7 mouse strains, C57BL and SJL, confirmed that isolate 2 readily passaged in s7s7 mice, with a comparatively short incubation period (dotted lines, Figure 2). The fact that the incubation period was significantly shorter in SJL mice than in C57BL mice is consistent with the data from a number of standard scrapie strains (Carp and Callahan, 1986) and was seen also in passage 2 of isolate 2 (Figure 2). The incubation periods of passage 2 in C57BL and SJL mice were similar to those found for the first passage in these mouse strains, suggesting that the incubation period values had stabilized.

Comparison of incubation period data, combined with other findings to be described subsequently, suggests that the characteristics of isolate 2 are easily distinguishable from those of all scrapie strains used in the laboratory, with the exception of ME7. To further assess this issue, the homogenate prepared from CW mice from passage 1 and an homogenate prepared from ME7-infected mice were injected by two investigators at the same time into C57BL mice. The results (Table 2) indicate that those groups injected with isolate 2 had significantly shorter incubation periods than the groups

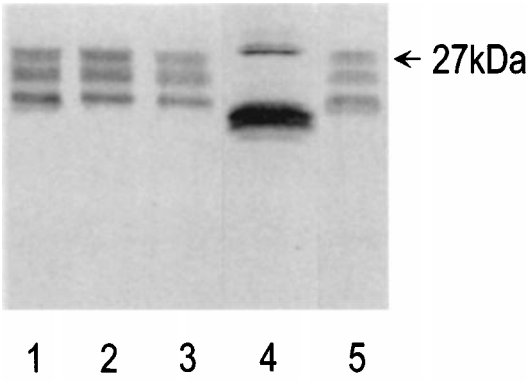

Figure 1 Western blot analyses of PrP^{Sc} from brains injected with mite homogenates. PrP^{Sc} was partially purified from mouse brains, as described in Materials and methods, following injection of mite homogenates (lanes 1–4) or the ME7 scrapie strain (lane 5). Lanes 1, 2 and 3 are from CW mice injected by either the i.c. or i.p. route and harvested at either 340 days or 376 days (Table 1, isolates 1, 2 and 3). Lane 4 is from a CW mouse injected i.c. and harvested at 501 days (Table 1, isolate 4). Equivalent quantities of protein were applied to each lane. Immunoreactivity was analyzed using the rabbit anti-mouse PrP^{Sc} antibody, 78295 (Kascsak *et al*, 1993).

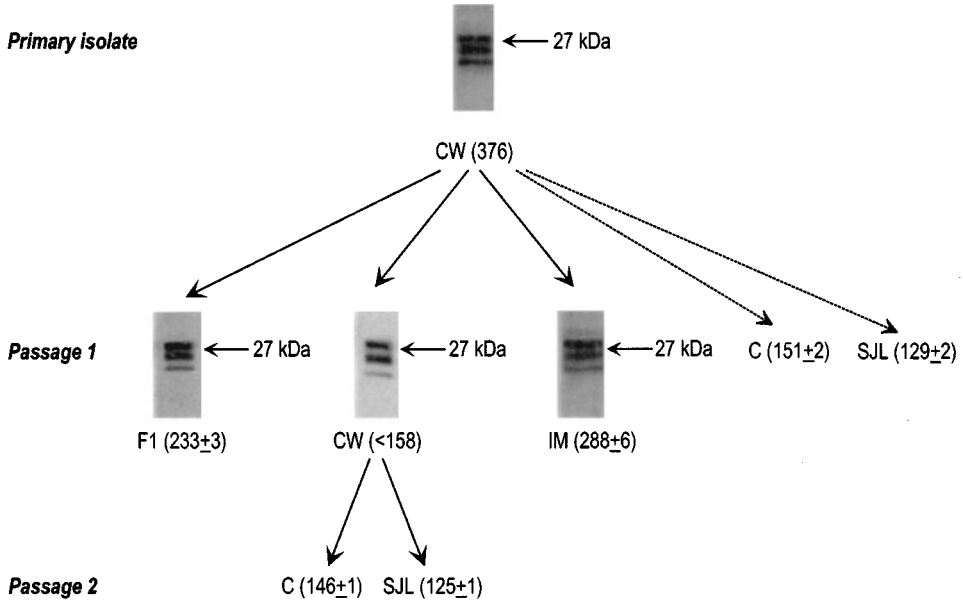

Figure 2 Flow chart of primary isolation, passage 1 and passage 2 of isolate 2. The chart depicts the WB profile and the incubation periods (mean ± s.e.) in parentheses. On the WB profiles, 27 kDa is indicated by the arrow. The first passages in C (C57BL) and SJL mice (dotted lines) were done subsequent to the first passages to F1, CW and IM mice (solid lines). WB profiles of C and SJL mice had the typical 3-band pattern at both first and second passage (data not shown). Number of mice in passages 1 and 2: all groups were nine or ten mice except the IM group which was six mice.

injected with the ME7 strain. The differences in incubation period between groups injected with the same inoculum by different investigators were not significant. The incubation period (146±1) in C57BL mice for passage 2 of isolate 2, noted in Figure 2, is similar to the values obtained for this isolate in the separate experiment noted in Table 2.

Homogenate prepared from the brain of the mouse injected with primary isolate 4 was assayed in the three Sinc genotypes and yielded incubation periods of 293, 439 and 515 in s7s7, s7p7 and p7p7 genotypes, respectively (Table 1, Figure 3). The first passage material from the s7s7 strain (CW) was used in a second passage into three s7s7 strains, CW, SJL and C57BL, and one p7p7 mouse strain, IM (Figure 3). The incubation periods in the three strains with the s7s7 genotype were all significantly shorter than the incubation period in IM mice. Thus, both groups of isolates, exemplified by isolates 2 and 4 in Figures 2 and 3, respectively, show a pattern similar to that of standard scrapie strains such as ME7 and 22L in which the incubation period in s7s7 mice is much shorter than the incubation period in p7p7 mice – an outcome that differs from the 22A, 87V group of scrapie strains in which the pattern is reversed.

Table 2 Comparison of incubation periods of isolate 2 and the ME7 scrapie strain in C57BL mice

	Isolate 2		*ME7*		
	Incubation period		*Incubation period*		
Investigator	n	*(day±s.e.)*	n	*(day±s.e.)*	P
A	10	147±3	9	161±2	<0.01
B	9	149±3	8	163±3	<0.01
A+B	19	148±2	17	162±2	<0.0001

Homogenates from the F1 and IM mice from passage 1 were also injected into both C57BL and SJL mice (see passage 2 of Figure 3). Interestingly, the incubation period data for second-passage mice injected with homogenates from F1 mice (s7p7→s7s7) were significantly shorter than the same mouse strains injected with homogenate material from CW mice (s7s7→s7s7).

Also shown in Figure 3 are the PrP^{Sc} WB profiles of brains from primary isolate mice and some of the mice from first passages. As noted above, for many mouse-adapted scrapie strains, e.g., ME7 and 22L, the PrP^{Sc} pattern after PK digestion yields three bands of approximately equal staining intensity at kDa values of 27–30, 24–25, and 21–22, corresponding to the diglycosylated, monoglycosylated and unglycosylated forms of the protein. The exceptions to this pattern have either a markedly diminished top band (the 139A strain) or a heavily stained top band and very little staining of the unglycosylated band (the 87V strain) (Kascsak *et al*, 1991). For isolate 4, the primary isolate (Figures 1 and 3) and the first passage in CW mice (Figure 3) yielded an aberrant pattern with only two bands, one slightly higher than the top band and the other slightly lower than the unglycosylated band. The PrP^{Sc} patterns for mice from the first passage into IM and F1 mice and for all mice at second passage were three equally stained bands. The reason for the unusual banding pattern from primary isolate and first passage CW mice is not known.

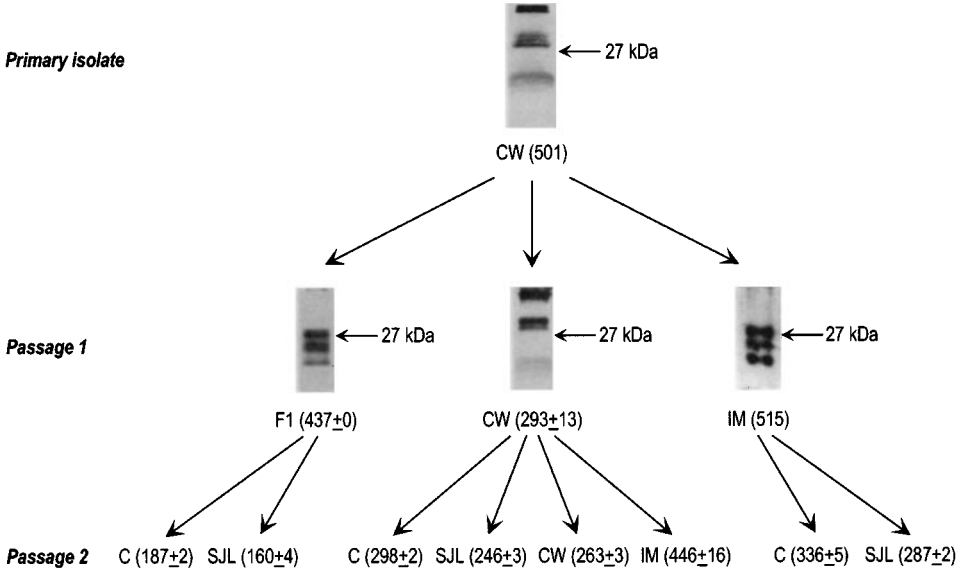

Figure 3 Flow chart of primary isolation, passage 1 and passage 2 of isolate 4. The chart depicts the WB profile and the incubation periods (mean±s.e.) in parentheses. On the WB profiles, 27 kDa is indicated by the arrow. WB of mice from passage 2 all revealed the typical 3-band pattern. C is C57BL. Number of mice in passage 1: F1=5, CW=5, IM=2; the number of mice in passage 2: CW=43, IM passage to C=3 and IM passage to SJL=3. All other passage 2 groups were 9 or 10.

The effects of isolates 2 and 4 on the body weight of SJL mice are shown in Figure 4. Each isolate caused a significant increase in body weight compared to mice injected intracerebrally with NMB. For each isolate, the increase in weight occurred in the latter part of the preclinical phase and obesity continued into the early portion of the clinical phase of disease. Similar findings were reported for the ME7 and 22L scrapie strains, whereas the 139A strain does not affect the weight of SJL mice (Kim *et al*, 1987).

Microscopic examination of brain from the primary isolate 2 mouse revealed diffuse vacuolation seen throughout the entire brain, from the olfactory bulbs to the medulla and cerebellum. Most of the vacuoles were rather small. They were seen distributed rather evenly throughout all cortical areas and basal ganglia (Figure 5a). Vacuoles were also seen in the cerebellar and brain stem nuclei. Some vacuolation was also seen in the white matter of the brain stem and cerebellum with some brain stem vacuoles appearing larger than those seen in supratentorial structures (Figure 5b). The vacuolation of the white matter was more extensive in the infratentorial rather than in the supratentorial structures. Histological changes were markedly more severe with isolate 4. The topography of vacuolation was similar to that seen with isolate 2. Vacuolation was seen throughout the entire central nervous system, but the diameter of the vacuoles was larger and the density was higher. The vacuolation appeared to be most extensive in the brain stem and cerebellum (Figure 5c,d). Although most of the vacuoles were small, there was a high number of medium-sized and large vacuoles, especially in the infratentorial structures. The white matter appeared to be markedly more involved with isolate 4 than with isolate 2. Subsequent passages of these isolates yielded mice with similar vacuolation patterns to those seen in primary isolate mice (data not shown).

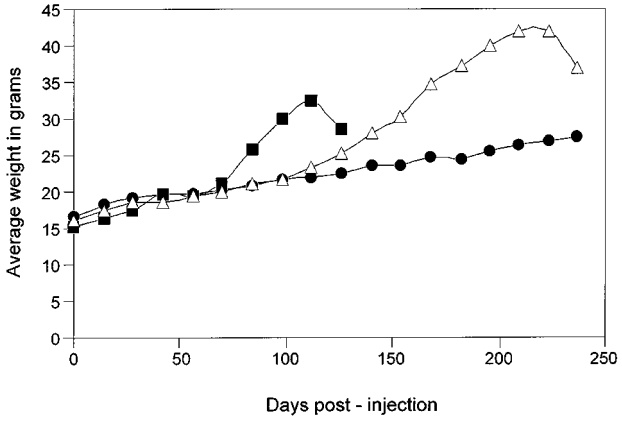

Figure 4 Average weight in grams of SJL mice injected with NMB, ●–●; isolate 2, ■–■; or isolate 4, △–△. Number of animals: NMB=9, isolate 2=10, isolate 4=9.

The characteristics of the four isolates were compared with those of the scrapie strains that are used in the laboratory (Table 3). The incubation periods for passage 1 of isolates 1, 2 and 3 in the three Sinc genotypes are similar to those of the ME7 strain, but differ from all other scrapie strains in one or more of the Sinc genotypes. It was shown, however, that isolate 2 differed from ME7: the incubation period of a second passage of isolate 2 in C57BL mice was significantly shorter than the incubation for ME7 in C57BL mice (Table 2). Isolate 4 differed from all strains with regard to the long incubation periods in mice of the three Sinc genotypes, the failure to cause motor dysfunction and the aberrant Prp^{Sc} profile in brain homogenates observed after primary isolation and first mouse-to-mouse passage in CW mice.

Discussion

The present study had two goals with regard to our previously reported isolation of infectious scrapie from hay mites. The first goal was confirmation of scrapie strain isolation by subsequent passages in mice. If the effects noted on primary isolation were a function of scrapie infection, then on subsequent passage, the effects should be consistent with the initial findings, and they should occur earlier. These criteria were fulfilled for the four primary isolates that were tested. The second goal was to determine the phenotypic characteristics of the isolates and to compare them with the phenotypic characteristics of various scrapie strains, particularly those used in the laboratory. The importance of the comparisons is that if isolates differ from scrapie preparations used in the laboratory, it provides compelling evidence that the isolates are not laboratory contaminants.

The results of the isolate characterization strongly suggest that these isolates from mites are different from the strains used in our laboratory. For isolates 1, 2 and 3, there were marked differences from a number of strains (Table 3), particularly with regard to incubation periods in mice of the three Sinc genotypes. The ME7 scrapie strain did have similar characteristics to those of isolates 1, 2 and 3 except that the incubation period of ME7 in C57BL mice was significantly longer than that of the second passage of isolate 2 in this mouse strain (Table 2); isolates 1 and 3 were not passaged beyond first passage. Isolate 4 differed from all of the scrapie strains with regard to a number of parameters; most striking were the very long incubation periods in mice of each of the three Sinc genotypes, the unique WB profile on primary isolation and on first passage in CW mice, and the unusual symptoms during the clinical phase of scrapie disease.

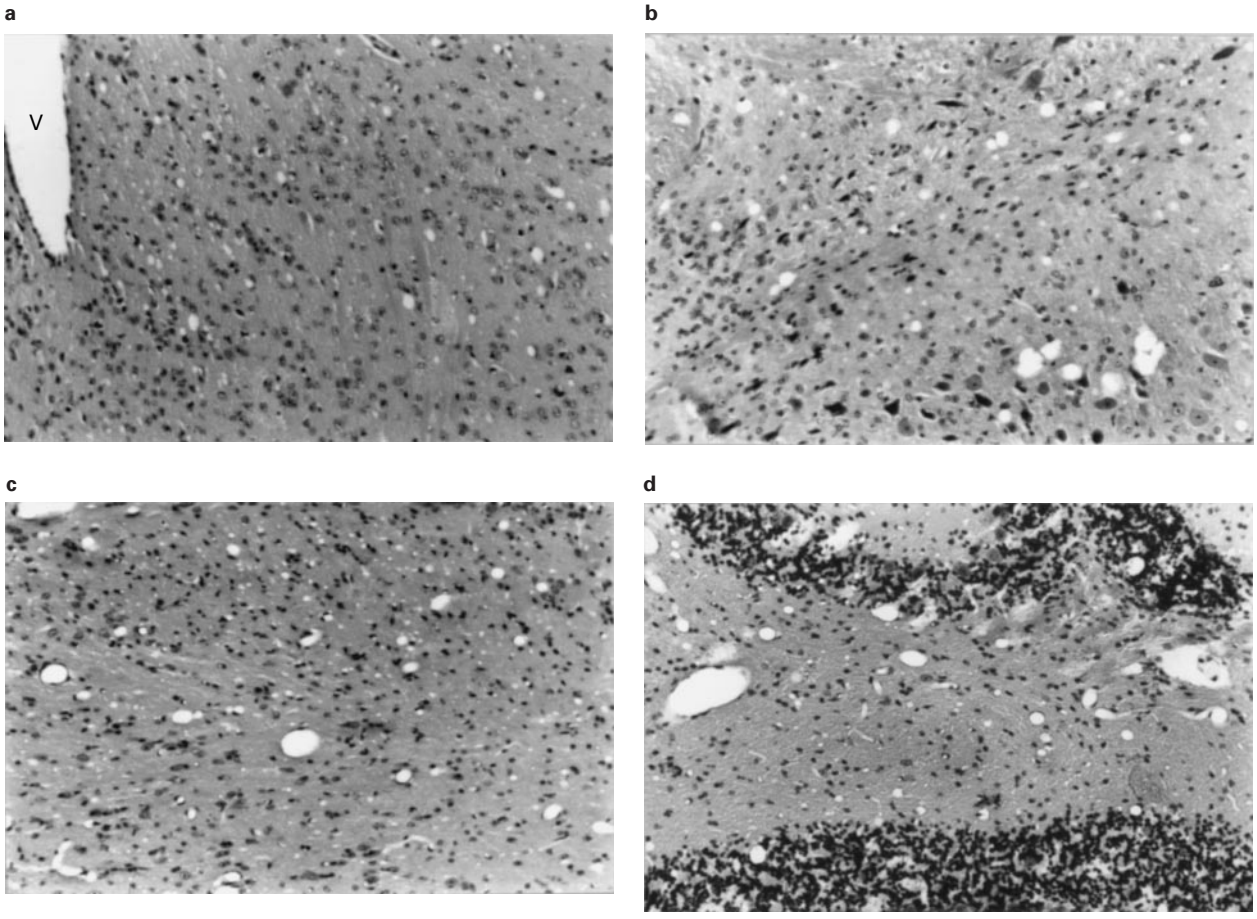

Figure 5 (**a**) Isolate 2: periventricular grey matter. Small vacuoles in the grey matter of the basal ganglia near the ventricle (v) Hematoxylin Eosin, ×400. (**b**) Isolate 2: midbrain. Small and large vacuoles seen mostly in the white matter of the long tract. Hematoxylin Eosin, ×400. (**c**) Isolate 4: midbrain. Numerous small vacuoles within the long tract of the midbrain. A few large vacuoles are also seen. Hematoxylin Eosin, ×400. (**d**) Isolate 4: cerebellum. Small- and medium-sized vacuoles in the cerebellar white matter. Vacuoles are scattered in between blood vessels. Hematoxylin Eosin, ×400.

Table 3 Comparison of the characteristics of five standard scrapie strains used in the laboratory to the characteristics of several mite isolates

	Isolate		*Scrapie strains*				
Characteristic	*2*	*4*	*ME7*	*22L*	*139A*	*22A*	*87V*
I.P.[(*)] C57BL (days)	<158	293	162	138	125	356	[(a)]
I.P. F1 (days)	233	437	220	160	170	484	[(a)]
I.P. IM (days)	288	515[(b)]	300	200	160	190	274
Obesity in SJL	+	+	+	+	−	−	−
Extensive vacuolation in forebrain	+	+	+	−	−	−	−
Motor deficits in clinical phase	+	−	+	+	+	+	+
Number of bands on WB	3	2[(c)]	3	3	3	3	3

[(*)]I.P.=incubation period; [(a)]Few, if any, mice developed scrapie, even after injection of high dose in brain; [(b)]Mice were clinically normal, but brains contained PrP^{Sc}; [(c)]Two bands on primay isolation and first passage in CW mice.

The unusual WB profile seen in CW mice at primary isolation was consistently found in multiple tests of the same brain homogenate. Furthermore, the unusual pattern was seen in three CW mice tested at the first mouse-to-mouse passage (Figure 3). Differences in WB profiles of brain preparations derived from mice injected with different scrapie strains have been reported previously (Kascsak *et al*, 1991). The surprising finding with regard to the data on isolate 4 was that this unusual pattern was not seen in first-passage IM or F1 mice or in several mouse strains at second

mouse-to-mouse passage (Figure 3). One possible explanation is that there are multiple scrapie strains in the original isolate that undergo different selective pressures in different mouse strains. The fact that second passages of homogenates from F1 mice into mice of the s7s7 genotype yielded significantly shorter incubation periods than second passages of homogenates from CW mice suggests that multiple scrapie strains are present in brain homogenate of isolate 4 following primary isolation. Further analysis of passages that originated from F1, IM and CW mice will be needed to establish the possibility that several strains are present.

As noted earlier, identical characteristics of mite isolates and a particular scrapie strain would not rule out a mite origin for the isolates. With regard to the characteristics of isolates compared to those of scrapie strains, the mite isolates fall into two categories. Category one, represented by isolates 1, 2 and 3, resembles scrapie strain ME7 used in our laboratory, although, as noted above, there was a significant difference between isolate 2 and ME7 with regard to incubation periods in C57BL mice. The methods used to prepare these pools were identical. This finding could be related to a fundamental difference in incubation periods or it could be a function of a titer difference between the brain homogenates used for injection; either cause would represent a difference between the mite isolate and ME7. Category two (isolate 4) displays characteristics that are unique. The incubation period data, the clinical manifestations and the WB results indicate that this isolate is different from any of the standard scrapie strains. Extension of these findings by positive isolations from additional scrapie-infected farms and by establishing that some of these isolates are unique would support the contention that mites contain infectious scrapie agent and therefore could act as a vector and/or reservoir for scrapie. This finding would impact on concepts of scrapie incidence and control. The ability of an agent to remain in an area without the presence of its natural host will greatly change our view of scrapie epidemiology. This, in turn, would impact on studies of the increasing incidence of chronic wasting disease in the western and central United States, the continuation of bovine spongiform encephalopathy in the United Kingdom, and the occurrence of sporadic CJD worldwide.

The presence of infectious scrapie in mite preparations would raise a number of questions concerning whether the mites can support scrapie replication, thus acting as a reservoir, or whether their role is that of a vector in which infectious agent is either actively sequestered by the mites or is adherent on their surface. It should be possible to answer these questions with appropriate mite populations maintained in a laboratory situation.

Materials and methods

C57BL/6J and SJL/J mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA), IM/DK mice were obtained from Alan Dickinson (Neuropathogenesis Unit, Edinburgh, UK) and have been maintained in our animal colony as an inbred strain. IM and C57BL x IM F1 mice were bred in our animal colony. Animals were fed and watered *ad libitum* and maintained in artificial light with a 12 h on, 12 h off cycle. Preparation and injection of mite samples (10% w/v) for primary isolation were described previously (Wisniewski *et al*, 1996). Subsequent passages were done by intracerebral (i.c.) injection of 25 μl of 1% (w/v) brain homogenates prepared as described previously (Carp *et al*, 1984). Mice were observed weekly for clinical symptoms on a grid apparatus used for monitoring motor coordination (Carp *et al*, 1984). Mice were sacrificed by intraperitoneal injection of 0.1 ml sodium pentobarbital (50 mg/ml). Brains were removed and sectioned longitudinally into two halves. One-half section of brain was placed in buffered paraformaldehyde for histopathology. The remaining half brain was divided approximately into one-third for PrP analysis and two-thirds for further passage.

The following mouse strains were used for analysis of scrapie incubation periods in different Sinc genotypes: Compton White (CW) mice have the s7s7 genotype, IM mice have the p7p7 genotype, and a cross between C57BL and IM mice provided the s7p7 F1 strain. The other mouse strains used for analysis were C57BL and SJL, which have the s7s7 genotype.

For analysis of obesity, mice were weighed just before injection and at 2-week intervals throughout the incubation period. The control group consisted of mice injected with 1% normal mouse brain homogenate (NMB).

For analysis of PrP^{Sc} by WB, 50–80 mg of coded frozen mouse brains were partially purified as described previously (Rubenstein *et al*, 1994), except that the treatment with proteinase K (100 μg/ml for 1 h at 37°C) was performed just before WB analysis. In brief, samples were homogenized in 10% sarkosyl and pelleted at 150 000 × g for 90 min using a Beckman TL 100 ultracentrifuge. Pellets were suspended in buffer containing 10% NaCl and repelleted (150 000 × g for 1 h) through a 20% sucrose cushion. Equivalent quantities of protein from each sample were electrophoresed on sodium dodecyl sulfate polyacrylamide gels (Laemmli, 1970). The proteins were transferred to nitrocellulose and immunostained (Rubenstein *et al*, 1994) with rabbit anti-PrP antisera (Kascsak *et al*, 1993).

Histopathological analyses were performed on specimens prepared and assessed by methods described previously (Carp *et al*, 1998).

For data analysis, the scrapie incubation period is defined as the time from injection until the third consecutive positive weekly clinical score. Differences in incubation period and induction of obesity were determined by analysis of variance (or the Mann-Whitney U-test for data requiring non-parametric analysis) using the Graphpad Software Version 1.12a package.

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