Biological Evidence that Scrapie Agent Has an Independent Genome

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SUMMARY

There are many distinct strains of scrapie agent, identified by their relative incubation periods and quantitative and qualitative neuropathological properties in inbred mice of particular genotypes. When serially passaged under specified conditions of mouse strain, route of infection and dose of infectivity these properties are stable. However, they may change in a predictable manner if the passage strategy is altered. The scrapie strain 87A shows what has previously been defined as Class III stability; it is stable when passaged at low dose in C57BL mice, but often suddenly changes its properties in the course of a single passage if high doses are used, always resulting in the same new strain. The latter, designated 7D, has shorter incubation periods and more extensive pathology than 87A, properties which are subsequently stable on serial passage even at high dose. This phenomenon has been seen repeatedly using scrapie isolates from six different natural cases in five different breeds of sheep. These isolates are closely similar in all their properties, showing them to be independent isolations of the 87A strain; there have been no isolations of 87A in which the phenomenon did not occur. On the other hand, none of the many other scrapie strains used in the same laboratory have shown this change. 87A brain samples consistently behave as if they contain 87A together with a smaller amount of 7D. This is so even after 87A has previously been passaged at high dilution, well beyond the limiting dilution for 7D, a procedure which would eliminate any minor agent strain originally present in the isolate. Therefore it is highly likely that the 7D in tissues of mice infected with 87A is generated de novo at each passage by mutational change from 87A during the incubation period. The established fact that many different strains exist and the considerable evidence that mutation can occur lead to the conclusion that scrapie agent has its own independently replicating genome.

INTRODUCTION

Mouse passage from a range of cases in sheep and goats has led to the identification of over 15 different strains of scrapie agent, distinguishable primarily on the basis of their incubation period properties in mice of defined genotypes. The major gene in mice controlling scrapie incubation period is the Sinc gene (scrapie incubation), two alleles of which have been identified. Using a standard dose and route of infection, each scrapie strain has a characteristic pattern of incubation periods in mice of the three Sinc genotypes (Dickinson et al., 1968; Dickinson & Fraser, 1977). In this laboratory most strain typing is performed in two inbred mouse strains, C57BL (SincsT) and VM (Sincp7), and in their F₁ progeny. This discrimination between scrapie strains is independently confirmed using quantitative pathological criteria, in particular the distribution of vacuolar degeneration in different parts of the brain as represented graphically by the lesion profile (Fraser & Dickinson, 1973). Scrapie strains also differ in a number of other respects, for example in the extent of cerebral amyloidosis produced (Bruce et al., 1976), their susceptibility to thermal inactivation (Dickinson & Taylor, 1978; Kimberlin et al., 1983), their neuroinvasiveness following peripheral infection (Dickinson & Outram, 1983) and the relative ease with which they can be transmitted from one species to another (Kimberlin & Walker, 1978). The obvious explanation for this considerable phenotypic diversity is that it reflects...
variation at the level of the informational molecule of the agent; the nature of this replicating informational molecule is not yet known, but a nucleic acid structure remains the most likely possibility.

When serially passaged in mice under specified conditions of dose, route and host genotype, scrapie strains have genetically stable properties. However, if the passage conditions are changed the properties may be altered, but these changes are themselves predictable. Scrapie strains have been grouped into three stability classes as follows (Bruce & Dickinson, 1979; Dickinson et al., 1984). Class I stability strains (e.g. ME7, 22C) are stable in their properties irrespective of the mouse genotype in which they are passaged. Class II strains (e.g. 22A, 22F) are stable when passaged in the Sinc genotype in which they were isolated but gradually change during serial passage in another Sinc genotype until a new set of stable properties is achieved; for any particular Class II strain these new properties have always been the same in replicate passage lines. Class III strains (e.g. 87A) often show a sudden discontinuous change in properties when passaged at high dose, even in the Sinc genotype in which they were isolated; so far this has always resulted in the same set of new properties, which are themselves subsequently stable on passage. The term breakdown has been coined to designate this type of event.

Changes associated with stability Classes II and III are interpreted as being due to mutations in the informational molecule of the scrapie strains involved, followed by host-permitted selection of mutants with shorter incubation periods in the particular mouse genotype used for passage. Mutation towards a longer incubation period has not been seen; such an event would be extremely difficult to detect, simply because the parent strain would kill the mouse before the mutant could replicate to high titre.

In this paper we present further evidence for the mutational basis of Class III stability, using data from six independent isolates.

METHODS

Scrapie isolates. The Class III isolates referred to in this paper originated from natural scrapie cases in different flocks, as follows: 87A was derived from a case in a Cheviot × Border Leicester sheep, 31A from a Dorset Down, 51C and 138A from separate Suffolks, 125A from a Southdown and 153A from a Scottish Blackface. The ME7 strain was originally isolated from a Suffolk sheep scrapie brain pool in another laboratory (Zlotnik & Rennie, 1963). Each isolate was maintained by serial intracerebral (i.c.) passage of 500 g supernatants of 10⁻² brain homogenates in C57BL mice. The 7D isolate used here was obtained by C57BL brain passage from a mouse with the characteristic short incubation period of 7D in an 87A experiment showing Class III breakdown at fourth passage. Short incubation derivatives of 31A, 51C and 138A were similarly obtained from experiments at third, fourth and third passages respectively. The results described in this paper are based on a large series of experiments which were carried out between 1965 and the present. During this time at least 15 other scrapie strains, with distinct properties from the strains mentioned above, were being handled in the same laboratory.

Mice. Two inbred strains of mouse, C57BL/FaBtDk (Sinc⁺) and VM/Dk(Sinc⁻), and the F₁ cross between these strains were used. Males and females were represented in approximately equal numbers.

Experimental details. For most experiments the inoculum was prepared from a small sample (usually about 10 mg) from the cerebral cortex of a terminally affected female C57BL mouse. Except for infectivity titration experiments (see below), the tissue was homogenized in physiological saline at 10⁻² dilution, using a Griffiths glass in glass manual grinder (10 strokes), and centrifuged at 500g for 10 min (all at room temperature). In general, 4- to 10-week-old mice were injected i.c. with 0.02 ml of inoculum. In a few experiments this standard design was varied as follows: (i) mice were injected with brain inocula by an intraperitoneal (i.p.) route or (ii) inoculum was prepared from spleen and injected i.c.

For infectivity titration experiments, small samples of brain were homogenized in saline, using high-speed shearing Teflon in glass homogenizers (10 strokes), centrifuged at 2000 g for 15 min and serial tenfold dilutions were injected i.c. into groups of C57BL mice. This was done firstly using 87A brain from the fifth C57BL passage. Three further 87A titrations were carried out, using terminally affected donors from high dilution groups in this first titration. In an additional titration in C57BL mice, 7D brain at the second passage after breakdown was used as the source of inoculum. For each titration the LD₅₀ infectivity titre was calculated by the Kärber method.

To compare 7D and ME7, each scrapie strain was injected i.c. into groups of C57BL, VM and C57BL × VM F₁ mice, using 10⁻² inoculum prepared from the brain of a terminally ill C57BL mouse. For 7D, the donor mouse had been infected at high dilution (10⁻⁵) in the above 7D titration. The ME7 was used at the 11th i.c. C57BL mouse passage and had previously been cloned by passaging at limiting dilution at the sixth and seventh passages, each
Table 1. Incubation periods of the six Class III stability isolates (based on experiments with no breakdown) and their C57BL-passaged short incubation period derivatives, in mice of the three Sinc genotypes, C57BL (Sinc\textsuperscript{a}), VM (Sinc\textsuperscript{pT}) and the F\texttextsuperscript{1} heterozygote, following i.c. injection of 10\texttextsuperscript{2} brain homogenates.

<table>
<thead>
<tr>
<th>Designation of isolate</th>
<th>Original isolate</th>
<th>Short incubation period derivative</th>
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<tr>
<td></td>
<td>C57BL</td>
<td>C57BL × VM</td>
</tr>
<tr>
<td>31A</td>
<td>331 ± 5</td>
<td>(446)</td>
</tr>
<tr>
<td>51C</td>
<td>349 ± 3</td>
<td>491 ± 8</td>
</tr>
<tr>
<td>87A</td>
<td>355 ± 5</td>
<td>513 ± 11</td>
</tr>
<tr>
<td>125A†</td>
<td>359 ± 6</td>
<td>(498)</td>
</tr>
<tr>
<td>138A</td>
<td>346 ± 4</td>
<td>(450 ± 18)</td>
</tr>
<tr>
<td>153A†</td>
<td>355 ± 6</td>
<td>(506 ± 2)</td>
</tr>
</tbody>
</table>

* For 87A, n = 15 to 43 animals per group; for all other isolates n = 6 to 20 per group, except for the values in parentheses where n = 1 to 3.
† Breakdown occurred using these isolates but further passage from short incubation period cases has not yet been carried out.

**RESULTS**

Table 1: Incubation periods of the six Class III stability isolates (based on experiments with no breakdown) and their C57BL-passaged short incubation period derivatives, in mice of the three Sinc genotypes, C57BL (Sinc\textsuperscript{a}), VM (Sinc\textsuperscript{pT}) and the F\texttextsuperscript{1} heterozygote, following i.c. injection of 10\textsuperscript{2} brain homogenates.

**Occurrence of Class III breakdown**

Class III breakdown was seen as a sudden shortening, in the course of a single mouse passage, of the incubation periods in all three Sinc genotypes, accompanied by a marked change in neuropathology. This phenomenon occurred repeatedly using six independent isolates of scrapie (31A, 51C, 87A, 125A, 138A and 153A), but was never seen with at least 15 other scrapie strains which were being used in a wide range of experiments in the same laboratory. Breakdown was detected in over 10 different inbred mouse strains and crosses, representing all three Sinc genotypes. However, for simplicity, only experiments in which it occurred in C57BL mice are described in detail in this paper.

On primary passage from sheep to mice the incubation periods for the six Class III isolates were very long (about 450 days for C57BL mice). At the next passage the incubation periods shortened and stabilized at the values given in Table 1 for the original isolate, except in experiments with breakdown (see below). For this reason data from the primary passage are excluded from the table. Very long incubation periods at first passage are seen in almost all transmissions of scrapie from one species to another and this is generally referred to as the species barrier. For the Class III isolates the pathology did not change between primary and subsequent passages. Therefore, the shortening in incubation period at second passage was quite distinct from Class III breakdown, which involved a change in pathology and could occur after several serial passages in mice. So far, the latest passage stage at which breakdown has been
The incubation periods and lesion profiles of the six Class III isolates were closely similar, within the range of variation expected for a single strain (Table 1, Fig. 1a), and all were associated with large numbers of cerebral amyloid plaques and a high frequency of focal asymmetrical vacuolation, as previously reported (Bruce & Fraser, 1982). Therefore, although the six isolates were obtained from unrelated natural sheep sources, including five different breeds, it is probable that they were independent isolations of the same agent strain. There were no other isolates with this set of properties which did not show Class III breakdown. In terms of breakdown all six isolates behaved in precisely the same way, as described below for 87A which was the most extensively studied of the group. On further passage in C57BL mice, the short incubation period derivatives of 31A, 51C, 87A and 138A were closely similar to each other (Table 1, Fig. 1b); further passage of the strains derived from breakdown of 125A and 153A has not yet been carried out.

The 87A isolate

Serial passage of the 87A isolate in C57BL mice by i.c. injection of $10^{-2}$ brain inocula gave rise to a strain with comparatively long incubation periods in the three Sinc genotypes (Table 1). In the majority of experiments these incubation period properties remained stable and could be maintained over many serial mouse passages (so far up to the eighth passage with no indication of a limit). However, in some experiments there were mice of each of the three genotypes with much shorter incubation periods, down to about 180 days for individual C57BL mice. This
occurred sporadically at all stages between primary and seventh passage, in about 40\% of experiments using 10^{-2} inocula. In most of these experiments only a proportion of the mice had short incubation periods (partial breakdown) but in some experiments all mice were involved (total breakdown). Serial i.c. passage in C57BL mice from short incubation period cases (< 300 days for C57BL donors) invariably led to the isolation of the new strain, 7D, which, on further passage, had stable incubation period properties quite distinct from those of 87A (Table 1).

The pathology in mice infected with 87A consisted of severe vacuolar degeneration confined to subcortical regions and showing a focal distribution. Vacuolation was consistently seen in the hypothalamus, dorsal medulla and ventral regions of the mesencephalon, especially in the medial raphe ventral to the aqueduct; remaining areas of brain were completely unaffected, apart from occasional foci which were often asymmetrically distributed (Bruce & Fraser, 1982). This distribution of lesions is reflected in the 'alpine' appearance of the lesion profile (Fig. 1a).

The pathology in mice with 7D was much more generalized, with diffuse symmetrical vacuolar lesions throughout most areas of the brain, giving a lesion profile which was very different from that of 87A (Fig. 1b). In addition, the frequency of cerebral amyloid plaques was about tenfold lower with 7D than with 87A. The pathological characteristics of 87A and 7D were, in fact, so different that individual mice could be classified unambiguously as having been affected by one strain or the other by examination of histological sections.

In experiments with partial or total breakdown of 87A using 10^{-2} brain inocula injected i.c., coded sections from individual C57BL mice were classified by two independent observers as having either 87A-type or 7D-type pathology on the above criteria (Fig. 2a). It was found that cases with 7D-type pathology had short incubation periods and cases with 87A-type pathology had long incubation periods, with a changeover at about 300 days; there was complete agreement between the two observers. The lesion profile for all of the 7D-type cases combined (Fig. 2b) was identical to the lesion profile for C57BL-passaged 7D, strongly suggesting that this was the strain responsible for producing clinical disease in these mice. Also, the profile for the 87A-type cases was very similar to the standard 87A profile, based on experiments with no breakdown (Fig. 2c).
Effect of dose of infectivity on breakdown

Breakdown was more frequent using higher doses of 87A, occurring in 100% of experiments with $10^{-1}$ i.c. inocula, 40% with $10^{-2}$ and 15% with $10^{-3}$. Four infectivity titration experiments were carried out using serial tenfold dilutions of 87A homogenates (Fig. 3). Breakdown was seen in three of these experiments (a, b and d). In each, the small brain sample used to prepare the inoculum behaved as if it contained a mixture of 87A and a smaller amount of 7D; however, an unusually high concentration of 7D was detected in one of these experiments (d), suggesting that in this case a focus of 7D had been included in the brain sample (see Discussion). Breakdown occurred in two of the experiments (b and d) using material from donor mice in high dilution groups ($10^{-4}, 10^{-5}$) in the first titration, well beyond the limiting dilution for 7D-type cases ($10^{-3}$) in this particular experiment (Fig. 3a). These donors also had incubation periods which were longer than the maximum incubation period for 7D injected i.c. at limiting dilution (Fig. 4a).

An 87A dose–response curve was constructed from the results of the above titrations; considering only cases with 87A-type pathology, the mean incubation period for each dilution group was plotted against the dose of 87A received by each mouse in the group. By excluding the cases with 7D-type pathology, the complication of a hybrid dose–response curve was avoided.
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This was compared with a dose–response curve derived from a titration of C57BL-passaged 7D (Fig. 4a). There was only a very small overlap between these two curves, at about 300 days, accounting for the division shown in Fig. 2(a) between cases with the two types of pathology.

For the Class III isolates other than 87A, breakdown was also seen in about 40% of experiments using 10⁻² i.c. inocula. Considering all six isolates together, a total of 37 separate experiments was performed, involving i.c. injection of 10⁻² dilutions of small brain samples from different donors. Of these, six experiments showed total breakdown, eight showed partial breakdown and 23 showed no breakdown. Using coded sections, individual mice were classified according to pathology type, and the mean incubation periods for 7D- and 87A-type cases were calculated for each experiment (Fig. 4b). The infectivity dose injected was estimated from these mean values by reference to the dose–response curves for 87A and 7D, as previously described for other models (Dickinson et al., 1969). This showed, firstly, that the 7D dose was consistently higher in experiments with total breakdown than in those with partial breakdown. Secondly, there was no consistent difference in the 87A doses between experiments with partial breakdown and those without breakdown (10³ to 10⁴ i.c. LD₅₀ units in 0.02 ml of 10⁻² inoculum). From these results it is possible to estimate the approximate relative titres of 87A and 7D in the majority of the large number of brain samples used as inocula. 7D was detected in 100% of 10⁻¹ inocula but in only 40% of 10⁻² inocula. Therefore the 60% of brain samples which did not yield 7D at 10⁻² would have contained between 1 and 10 i.c. LD₅₀ units of 7D in a 10⁻¹ dose, compared to 10⁴ to 10⁵ i.c. LD₅₀ units of 87A.

Comparison of 7D and ME7

At least 15 different scrapie strains have been characterized in this laboratory; of these 7D most closely resembled ME7. A fully coded comparison of ME7 and 7D in C57BL, VM and C57BL × VM F₁ mice revealed no significant differences between incubation periods in any of the genotypes tested (Table 2). Lesion profiles for the two scrapie sources were also very similar (Fig. 5). On the basis of these results it was not possible to distinguish between the two and it is therefore probable that 7D and ME7 are independent isolations of the same strain.
Table 2. Coded comparison between the incubation periods of 7D and ME7 in mice of the three Sinc genotypes, C57BL (Sinc07), VM (Sincp7) and the F1 heterozygote, following i.c. injection of 10^-2 brain homogenates

<table>
<thead>
<tr>
<th>Scrapie strain</th>
<th>C57BL</th>
<th>C57BL x VM</th>
<th>VM</th>
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<tbody>
<tr>
<td>7D</td>
<td>169 ± 2</td>
<td>246 ± 1</td>
<td>321 ± 4</td>
</tr>
<tr>
<td>ME7</td>
<td>171 ± 2</td>
<td>251 ± 2</td>
<td>326 ± 3</td>
</tr>
</tbody>
</table>

* n = 12 to 18 animals per group.

Effect of route of infection and organ source of inoculum

Breakdown of Class III isolates was not confined to experiments using i.c. injected brain inocula. It was also seen in four out of seven experiments in which spleen inoculum was injected i.c. and in four out of ten experiments in which brain inoculum was injected i.p., in each case using homogenates in the dilution range 10^-2 to 5 \times 10^-2. The pattern was similar to that for i.c. injected brain, except that with the i.p. route the changeover between 7D-type and 87A-type cases was about 450 days. This was to be expected since for both scrapie strains the incubation period following i.p. infection is about 50% longer than the i.c. incubation period with the same inoculum.

DISCUSSION

The six Class III stability isolates consistently behave like mixtures of a long incubation period strain accompanied by smaller amounts of a short incubation period strain. The crucial questions are how and at what point in the passage history this mixture arises. The simplest explanation is that the minor component is derived by mutational change from the long incubation period strain originally isolated. However, two other possibilities should first be excluded.

The breakdown product of 87A, 7D, is indistinguishable from ME7. It is likely that ME7 itself was derived by breakdown from a Class III isolate in another laboratory. In the first mouse passage of that isolate, vacuolation was reported to be confined to the brainstem, further passage at high dose resulting in a change to a more generalized type of pathology and a shortening of the incubation period (Zlotnik & Rennie, 1967); this change was not seen when the dose of infectivity was considerably reduced by boiling the inoculum. ME7 has been used extensively in
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our own laboratory for many years, raising the possibility that breakdown is actually the result of repeated contamination of more recently isolated strains with ME7. This cannot be the case for the following reasons. Class III breakdown has only been seen using the six isolates described here (plus the original ME7 isolate) which have closely similar properties and are almost certainly the same strain. In the case of the most recently isolated of these, 153A, it was even possible to predict that it would have Class III stability, simply from the biological and pathological properties at primary passage, when breakdown did not occur. During the 20 years in which strain typing has been carried out in this laboratory breakdown has never occurred using at least 15 other distinct strains, even though some of these strains in particular mouse genotypes have extremely long incubation periods which would ensure the detection of small contaminating doses of an operationally short incubation period strain. Furthermore, the breakdown product has always been indistinguishable from ME7 and unlike any of our other short incubation period strains.

Another possible alternative to mutation is that 87A and 7D were already both present in the naturally affected sheep and were isolated and passaged in mice as some type of stable mixture. However, given the large difference in incubation periods between the two strains and the fact that their dose–response curves hardly overlap, it is extremely unlikely that such a mixture could survive serial passage without 7D rapidly becoming the predominant strain, unless 87A suppresses its pathogenesis in some unknown way. It is well established that a long incubation period strain can delay or block the subsequent pathogenesis of a short one, probably by occupying replication sites (Dickinson et al., 1975; Dickinson & Outram, 1979; Kimberlin & Walker, 1985) or replication initiation sites (R. G. Rohwer, personal communication), but this has only been observed when the two strains were injected at a considerable time apart; it has never been seen when the strains were mixed in the same inoculum. Finally, the most compelling evidence that 7D is not simply emerging from a long-maintained mixture is that the ability of 87A to break down survives passage at high dilution, well beyond the limiting dilution for 7D, indicating that 7D is being generated de novo from 87A during each 87A passage. Therefore breakdown is the result of a change in the information carried by the agent, rather than the sudden emergence of a minor component from a pre-existing mixture.

The basis of such an informational change could either be mutation or some type of heritable host-induced modification of the agent. While it is possible that host-dependent changes might contribute to species barrier effects when scrapie is transmitted from one species to another, it should be emphasized that in the present study the host species and genotype (C57BL) are held constant. Bearing this in mind, the fact that 87A can maintain its properties indefinitely when passaged at low dose argues convincingly against any host-induced changes being involved in breakdown. We therefore strongly favour the conclusion that the change from 87A to 7D is a conventional type of mutational event resulting in a mutant strain which is at a selective advantage because of its shorter incubation period.

In most small samples of terminal 87A brain tested, the ratio of 7D to 87A was of the order of 1 : 10^4. The actual mutation rate is likely to be lower than this because 7D units generated by mutation throughout the incubation period would be predicted to replicate faster than the parental strain. The final composition of the mixture would depend not only on the mutation rate but also on the complexities of replication dynamics of the two scrapie strains in different parts of the brain. Occasional small brain samples were found to contain a much higher concentration of 7D, in some cases approaching that of 87A (Fig. 3d and 4b), suggesting that 7D is focally distributed in the brain. Focal asymmetrical vacuolar lesions are a striking feature of the pathology of the Class III group of isolates (Bruce & Fraser, 1982). They can occur in many areas of the brain but are most common near the site of the i.c. injection. It is possible that these lesions are the pathological consequence of focal replication and accumulation of 7D, derived by mutation from a single 87A agent copy relatively early in the incubation period. By chance, the small cortical sample used for preparing the inoculum could occasionally contain such a focus, thus accounting for the instances in which unusually high titres of 7D are encountered.

The molecular nature of the scrapie agent is not yet known. The many attempts to link infectivity with any particular molecular species have been inconclusive, although there is
longstanding agreement that protein is required for infectivity. A 27K to 30K host-coded protein has recently been identified in brain extracts from scrapie-infected animals (McKinley et al., 1983; Oesch et al., 1985; Chesebro et al., 1985), but claims that this protein is an integral part of the infectious agent are open to doubt (Kimberlin, 1986). No specific nucleic acid has yet been identified in tissue fractions which are rich in infectivity. This and observations that infectivity is protease-sensitive but nuclease-resistant have renewed the speculation that the agent itself is an infectious protein devoid of nucleic acid (Lewin, 1981; Prusiner, 1982; German & Marsh, 1983). The major objection to the idea of an infectious protein is the existence of many different strains of scrapie, the replication of each being specific and exclusive, apart from the predictable occurrence of mutation. All of the hypothetical replication mechanisms put forward for an infectious protein become highly implausible when confronted with these facts.

The considerable strain diversity in scrapie, together with the evidence for mutational change presented here and elsewhere (Bruce & Dickinson, 1979; Dickinson et al., 1984), offer compelling arguments that scrapie has its own independently replicating genome. We consider that the informational molecule of scrapie is most likely to be a small, as yet undetected, nucleic acid which is protected, either specifically or non-specifically, by host tissue components (the virino hypothesis detailed by Dickinson & Outram, 1983). If scrapie agents contain a nucleic acid which is responsible for strain diversity, then it should, for example, be possible to modify mutation rates in scrapie by using treatments which are mutagenic for conventional viruses.

REFERENCES


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