Scrapie strains retain their distinctive characteristics following passages of homogenates from different brain regions and spleen

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The molecular basis of differences among scrapie strains is unknown. The prion theory posits that there are differences in the conformation of the host protease-resistant protein (PrP) molecules and that these differences are responsible for scrapie strains. A corollary of this theory is that the origin of host PrP variation resides in different neuronal cell types. To assess this concept, preparations from three brain regions (cerebrum, cerebellum and olfactory bulb) and from spleen were passaged in C57BL mice by intracerebral injection. After three passages of three scrapie strains in this manner, homogenates of each brain region and spleen were tested for several of the characteristics that distinguish the three strains: (1) the rank order of incubation periods in C57BL mice, (2) induction of obesity in SJL mice and (3) comparative incubation periods in mice with three genotypes for the scrapie incubation period marker. Analysis revealed that virtually all of the criteria that distinguished the three strains prior to passages of the three brain regions and spleen were retained after this series of passages. This finding argues against cellular-based PrP differences providing a basis for strain specificity.

Introduction

In the continuing discussion of the nature of scrapie agent, a critical question is how the agent replicates and at the same time maintains the phenotypic distinctiveness that characterizes a variety of scrapie strains (Dickinson & Outram, 1983; Carp et al., 1987; Carp & Rubenstein, 1991). An auxiliary question that then arises concerns the origin of the molecular differences that yield scrapie strains. Those hypotheses, virus and virino, that posit a nucleic acid as the informational component of the prion hypothesis suggest that the original strain yields a nucleic acid sequence. In contrast, the prion hypothesis (Prusiner, 1982, 1991) explains the dual phenomena of phenotypic continuity and phenotypic variation in terms of the molecular biology of a single protein, the host-encoded protein commonly referred to as prion protein, or of its scrapie-induced form, protease-resistant protein (PrPSc) (Bolton et al., 1982; Prusiner et al., 1990; Weissman 1991a, b). The current concept of replication is that the incoming scrapie-specified protein (PrPSc) changes the tertiary structure of the normal host PrP molecule yielding a change identical to that of the ‘infecting’ PrPSc molecule (Prusiner, 1991; Cohen et al., 1994; Priola & Chesebro, 1995). According to this concept, slight differences in the molecular morphology of PrPSc molecules would provide the molecular basis for different scrapie strains; these structural differences would be manifested in the secondary and/or tertiary structure of the ‘progeny’ molecules. Proponents of the prion hypothesis suggest that the original strain variation develops and then is maintained because of slight differences in the normal PrP molecule that are present as a function of location within the brain and/or the type of PrP within infectible cells (Carlson et al., 1994). Differences in the characteristics of populations of scrapie agent would be a function of the different cell types affected by the infecting agent during the previous passage of the strain. For example, the neuronal cells of the cerebellum might provide a PrP molecule that would be particularly susceptible to conversion to PrPSc by a specific structure of the infecting PrPSc molecule (scrapie strain?). Furthermore, this conversion would yield a structure identical to that of the incoming PrPSc.

After the prion hypothesis was formulated, new data on the molecular biology of PrP required modification of the prion concept with regard to how the agent replicated as well as the origin and maintenance of strain differences (Prusiner, 1982; Prusiner et al., 1990; Weissman 1991a, b). In one paradigm, passage of scrapie strains from specific brain regions and/or spleen would...
reveal if strain characteristics were dependent upon the cellular types that produced the ‘infecting’ PrPSc (Carp et al., 1994[b]).

In the current study, incubation periods in different inbred mouse strains and the capacity to induce obesity were analysed in mice injected with three scrapie strains after passages using preparations of three brain regions or spleen as inoculum. For the three strains, virtually all of the previous analyses of phenotypic expression of incubation periods and obesity markers have been done using homogenates prepared from whole brain or small slices of cerebrum. In the current experiments, passages were done and pools prepared using homogenates of cerebrum, cerebellum, olfactory bulb or spleen. These preparations were then analysed for some of the markers that distinguish the three scrapie strains.

**Methods**

- **Animals.** Female weaning SJL/1 and C57BL/6 mice were obtained from Jackson Laboratories. 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 × IM F1 mice were bred in our Animal Colony. Animals were fed and given water *ad libitum* and maintained in artificial light with a 12 h on, 12 h off cycle. In obesity studies, mice were weighed on the day before injection, once every two weeks for 99 days post-injection (p.i.) and once a week from day 106 to 245 p.i. (conclusion of experiment). Animals were observed weekly for clinical symptoms by evaluating their movement on a grid apparatus that is useful for monitoring motor coordination (Carp et al., 1984). Mice were sacrificed by euthanasia in a CO2 box; brains, brain regions and spleens were harvested for passage.

- **Inocula.** The ME7 and 22L mouse-adapted strains were provided by Alan Dickinson (Neuropathogenesis Unit, Edinburgh, UK). The 139A strain was provided by Richard Kimberlin (Neuropathogenesis Unit, Edinburgh, UK). These strains were passaged and maintained in our laboratory as previously described (Carp et al., 1984, 1987). Homogenates (whole brain, brain regions and spleen) were prepared as 1% (w/v) suspensions in PBS, and 0.025 ml samples injected into mice intracerebrally (i.c.). Control mice were injected with homogenates of brain (NMB) or spleen (NMS) prepared from normal mice. For the first injection, whole brain homogenates of 139A, 22L and ME7 were injected into C57BL mice. ‘Early’ spleens were harvested from these mice sacrificed at day 73 p.i., and ‘late’ spleens and brain regions (cerebrum, cerebellum and olfactory bulbs) were harvested from all other mice at the end of scrapie incubation. Early spleen, late spleen and brain regions were then passaged three times in C57BL mice before final injection into SJL mice for obesity studies and into C57BL, IM and C57BL × IM F1 mice for incubation-time studies. At each passage, mice injected with a specific brain region or spleen harvest (early or late) provided the same brain passages.

**Results**

**Incubation periods in C57BL mice of cerebrum, cerebellum, olfactory bulb and spleen passages of scrapie strains 139A, 22L and ME7**

A typical example of the pattern of incubation periods of unpassaged 139A, 22L and ME7 whole brain homogenates is shown in Fig. 1 (set of columns on the left). The incubation period of the 139A strain was the shortest, of the 22L strain, intermediate and of ME7, the longest. This same pattern was seen for cerebrum, cerebellum and olfactory bulb homogenates of the three scrapie strains for the first passage in C57BL mice; with one exception, the pattern was retained for each region during the second and third passages (Fig. 1). The only exception was the second passage of olfactory bulb homogenates, in which the 22L strain yielded a slightly longer incubation period than ME7; the incubation period of the 139A strain was again the shortest for both the second and third passage material, regardless of the brain region used. The incubation periods tended to become slightly longer with succeeding passages.

The incubation period data for spleen passages are also shown in Fig. 1. In addition to homogenates prepared from spleens obtained at the end of the incubation period, spleens were also harvested at 70–102 days p.i. The use of preparations of spleens harvested early was included because of the possibility that early and late populations of scrapie agent in spleen may differ. In this context, it should be noted that peak titres in spleens are reached at the time of the early harvest, and the spleen titre of infectivity remains relatively constant throughout the rest of the incubation period (Rubenstein et al., 1991). Thus, it is possible that the early harvest is actually infectivity that had been produced recently, whereas the late-harvest infectious agent may have been present in the spleen for a considerable time. The groups of spleen preparations are referred to as ‘early’ and ‘late’. Of the three strains, 139A had the shortest incubation period at each passage for both early and late preparations (Fig. 1). The ME7 and 22L strains did not yield a consistent relationship with each other: for early spleen preparations, the 22L strain had the longest incubation period.
Scrapie strain stability following passages

Fig. 1. Scrapie incubation periods in C57BL mice of brain homogenate prior to passage and of passaged cerebrum, cerebellum, olfactory bulb, early spleen and late spleen preparations of scrapie strains 139A (filled bars), 22L (open bars), and ME7 (hatched bars). Numbers under the bars indicate passage number.

for passages 1 and 2, but not for the third passage. For late-harvested material, the incubation period for 22L was the longest for the first passage, but the ME7 strain had the longest incubation period for the second and third passages. A fourth passage into C57BL mice was part of the protocol used for the site-specific study noted in Fig. 4: the incubation periods for 139A spleen homogenates were again the shortest, whereas the incubation periods for 22L and ME7 were virtually identical.

The capacity of passaged scrapie strains 139, ME7 and 22L to induce obesity in SJL mice

Previous studies with the three strains using whole brain or spleen homogenates prepared from C57BL mice demonstrated that the ME7 and 22L strains induced obesity in SJL mice during the preclinical and early clinical periods; in contrast, the body weights of 139A-injected SJL mice were similar to those of mice injected with normal mouse brain homogenate (Carp et al., 1984; Kim et al., 1987, 1988). For the current study, homogenates of the three brain regions and spleen were prepared from the third sequential passage in C57BL mice. The results in Fig. 2 show that the capacity to induce obesity was retained by the cerebral-, cerebellar-, olfactory bulb- and spleen-passaged material from the 22L and ME7 strains, whereas preparations from passages of the 139A strain failed to cause a weight increase. For brain regions, body weights were compared with those observed for mice injected with NMB. The curves shown in Fig. 2(a–c) represent those derived from best-fit equations for each group. The results showed that for the ME7- and 22L-injected groups, the curves had a significantly higher ($P < 0.001$) slope and greater ($P < 0.001$) curvilinearity, regardless of the brain region used as inoculum. In contrast, growth curves for mice injected with brain regions from the 139A strain did not differ significantly from the curve for mice injected with NMB.

Data obtained for SJL mice injected with homogenates from the third passage of spleens harvested late after infection are shown in Fig. 2(d). Again, the curves represent the best-fit equation for each group. The linear trends of the curves for ME7 and 22L spleen preparations did not differ significantly from the normal spleen-injected group; however, the curves derived from the two scrapie strains did differ significantly in their curvilinearity. It is also obvious that the maximum mean weights attained by mice injected with the third passage of ME7 and 22L spleen homogenates were much higher than for mice injected with normal spleen. Values for maximum weight for 139A spleen preparations did not differ significantly from mice injected with NMS and the 139A and NMS curves did not differ in their slopes or curvilinearity.

All of the above data from spleen homogenates were obtained with spleens harvested late in the incubation period. Spleens harvested early during passages were also tested after
the third passage. The data (not shown) were very similar to those noted above, with spleen passages from ME7 and 22L strains causing significant increases in body weight, whereas the values for 139A-infected mice were not significantly different from those obtained for mice injected with normal spleen.

The comparative patterns of incubation periods of 139A, ME7 and 22L in SJL mice were similar before and after passage of specific brain regions. Prior to passage, brain homogenates of the 139A strain had the shortest incubation period in SJL mice, whereas incubation periods for ME7 and 22L were equivalent and approximately 11 days longer (Fig. 3); following three sequential passages of the three brain regions in C57BL mice, the incubation periods in SJL mice yielded patterns similar to those seen for whole brain prior to the passages (Fig. 3). Incubation periods for ME7-derived cerebrum, cerebellum and olfactory bulb preparations were similar to those for 22L, whereas preparations of passages of 139A brain regions were significantly shorter. For spleen-passaged preparations, the order of incubation periods from shortest to longest was 139A, ME7 and 22L for homogenates prepared from those spleens harvested both early and late in the incubation period (Fig. 3).

### Incubation periods of the third C57BL passages of brain regions and spleen of scrapie strains ME7, 22L and 139A in mouse strains with the s7s7, s7p7 and p7p7 genotype

Another difference among these scrapie strains that could serve as a marker is their comparative incubation periods in s7s7, s7p7 and p7p7 mouse strains. Previous studies using whole brain homogenate preparations showed the following patterns: the ME7 and 22L strains had the shortest incubation periods in C57BL mice (s7s7) and the longest incubation periods in IM mice (p7p7), whereas incubation periods were intermediate in C57BL × IM F1 mice (s7p7) (Dickinson & Meikle, 1971). In contrast, for the 139A strain, the longest incubation periods were in F1 mice, and the intermediate incubation period was in the p7p7 strain, a phenomenon referred to as overdominance (Dickinson & Fraser, 1979; Carp et al., 1987). In the context of scrapie incubation periods, overdominance is manifested by a longer incubation period of...
Scrapie strain stability following passages

Fig. 3. Scrapie incubation periods in SJL mice of brain homogenate prior to passage and of the third C57BL passage of cerebrum, cerebellum, olfactory bulb, early spleen and late spleen preparations of scrapie strains 139A (filled bars), 22L (open bars) and ME7 (hatched bars).

![Graph showing incubation periods for different brain regions and passage times for strains 139A, 22L, and ME7.]

Fig. 4. Scrapie incubation periods of the third C57BL passage of cerebrum, cerebellum, olfactory bulb, early spleen and late spleen preparations of scrapie strains ME7, 22L and 139A in C57BL (C), IM (I) and C57BL × IM F1 (F) mice.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Cerebrum</th>
<th>Cerebellum</th>
<th>Olfactory bulb</th>
<th>Early spleen</th>
<th>Late spleen</th>
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<tr>
<td>139A</td>
<td>C</td>
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<td>I</td>
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<td>22L</td>
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<td>ME7</td>
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The only samples that yielded a different pattern were the passages of 139A spleen homogenates in which incubation periods for the third passage for both early and late harvests were shortest in C57BL, longest in IM and intermediate in the F1 hybrid mice (Fig. 4).

a scrapie strain in F1 hybrid (s7s7 × p7p7) mice than in either parental strain. In the current study, the patterns noted above were retained by the cerebrum, cerebellum and olfactory bulb preparations for the three scrapie strains and by early and late spleen homogenate preparations from ME7 and 22L (Fig. 4).
Discussion

In previous studies, differences among scrapie strains have been maintained during serial passages in a single inbred mouse strain using homogenates prepared from whole brain (Carp & Rubenstein, 1991; Carp et al., 1994a, b). In order to assess the role of different cell types, we modified the procedure by using homogenates of specific brain regions or spleen as the inoculum. Following three passages, the homogenates prepared from the three brain regions and spleen were assayed for three phenotypic characteristics: (1) the rank order of incubation periods in C57BL mice, (2) the capacity to induce obesity and (3) the comparative incubation periods in s7s7, p7p7 and s7p7 mouse strains. For most of the assays the results of passages of brain regions and spleen were similar to those of the original whole brain and spleen homogenates. For example, for the obesity marker, the preparations of 22L and ME7 all caused obesity in SJL mice during the latter part of the incubation period and the early clinical phase of disease, whereas preparations of 139A did not cause a consistently significant increase in weight when compared to mice injected with NMB or NMS.

There were two differences from the findings observed with the original preparations of inoculum. In incubation period analyses of passaged spleen homogenates in C57BL mice, the first and fourth passages of ME7 and 22L were in the same rank order as the original unpassaged spleen, with ME7 shorter than 22L: however, this pattern was reversed for the second and third passages. The other different finding was obtained with 139A spleen passages in comparisons of incubation periods in s7p7 and p7p7 mice. For spleen-passaged preparations, incubation periods for F1 mice (s7p7) were intermediate between those for C57BL and for IM mice (p7p7), whereas for the original whole brain homogenate and for passaged brain regions of 139A, the incubation periods for F1 mice were longer (overdominance?) than those for IM mice. Overdominance was originally said to apply to several scrapie strains, including 139A (Dickinson & Fraser, 1979). Subsequent studies suggested that the findings for 139A did not reflect true overdominance, but rather that the F1 incubation period findings were related to the fact that many preparations of 139A are composed of two strains, with one strain, termed 79A, causing disease in s7s7 homozygotes and in heterozygotes, and the other scrapie strain causing the disease observed in p7p7 homozygotes (Dickinson & Outram, 1983). With this assumption, the explanation of the incubation period findings for spleen-passaged 139A is that in the mixture of strains, the strain that causes disease in p7p7 mice does not replicate well in spleen tissue and that the pattern of incubation periods obtained with spleen preparations is that of the other scrapie strain (79A). Current studies are directed toward establishing whether the 139A preparation that we have used is composed of more than one strain.

The preponderance of data obtained with passaged brain regions and spleen was similar to that obtained with the original material, suggesting that different neuronal cell types and even different organs did not provide information that could form the basis for the phenotypic differences observed among these three scrapie strains. If differences in PrP molecules were responsible for the distinctive phenotypic expression of these strains, the use of a specific brain region or spleen preparation should have eliminated or at least markedly reduced the differences. For example, the incubation periods of 139A were shorter than those of the other strains, for both original preparations and for those that had been passaged using spleen or specific brain regions as inoculum. In previous studies using dilutions of brain homogenates, differences in the incubation periods of the 139A and ME7 strains were observed even when mice were injected with equivalent doses of agent (Carp & Callahan, 1986); this was observed with two routes of injection (i.c. and i.p.), with two doses of each scrapie strain and in three mouse strains (C57BL, SJL and NZW). This finding argues against the shorter incubation period of 139A being a function of higher titres of homogenates.

The neuronal cell populations found in the three brain regions used to produce infectious agent in this study are clearly different. If one accepts the concept that scrapie replicates in a neuronal cell type within the brain (Kretzschmar et al., 1986; DeArmond et al., 1987), then the divergent characteristics of the three scrapie strains cannot be a function of the postulated differences in PrP molecules produced in different neurons in various brain regions. It is possible that the distribution of a specific susceptible neuronal cell type is widespread, i.e. the cell type occurs in the three different brain regions tested. If a single ubiquitous cell type is susceptible, then neuronal-specified differences in PrP should not be a factor in determining scrapie strains. Whether the distribution of neuronal cell types susceptible to specific scrapie strains is wide or narrow, passage of different scrapie strains from a single region should lead to a convergence of the characteristics of the strains. This did not occur. If scrapie replicates in non-neuronal cells within the brain (Diedrich et al., 1991; Carp et al., 1993), then the postulated differences in these cells from region to region (Lawson et al., 1990; David & Ness, 1993) would also not provide the basis for PrP-induced strain variation, since different regions were used in the present study without affecting strain characteristics. Furthermore, the fact that in most tests spleen-passaged material retained the strain-specific markers seen with brain argues against a role for differences in neurons or neuronal support cells in the induction of the specificity of scrapie strains.

The distribution of PrPsc differs as a function of both scrapie strain and infected host (Bruce et al., 1989; Jendroska et al., 1991; Hecker et al., 1992; Taraboulos et al., 1992; Casaccia-Bonnefil et al., 1993). The distribution of histopathological changes such as vacuolation and astrocytosis was similar to the pattern of PrPsc development (Bruce et al., 1989; Casaccia-Bonnefil et al., 1993; DeArmond et al., 1993). The site-directed
changes seen in scrapie have been referred to as targeting (Kimberlin & Walker, 1983; Bruce et al., 1989; Carp et al., 1994b). The basis of targeting in scrapie could be related to differences in PrP molecules in different cell types, but there is no evidence for this, and at this time the cause of the exquisitely precise targeting of histopathological changes in scrapie remains to be discovered.

In most cases, dramatic differences in phenotypes have no demonstrable basis in the physical, chemical or immunological characteristics of PrP molecules found in preparations of different strains (Carp & Rubenstein, 1991; Kascak et al., 1991). In several instances, differences in PrP between scrapie strains and between strains of transmissible mink encephalopathy have been demonstrated; these could provide a possible explanation for the specific characteristics that distinguish the strains (Kascak et al., 1991; Bessen & Marsh, 1992). However, even in these limited examples, it has not been proven that these differences are the direct cause of the phenotypic variation.

Results of this study support the opinion that the origin of scrapie strain differences remains to be determined.

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