Pathogenesis and Pathology of Scrapie after Stereotactic Injection of Strain 22L in Intact and Bisected Cerebella

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Abstract. The mechanisms involved in the spread of scrapie within the brain remain unclear. To examine this issue the 22L scrapie strain was injected in one side of the cerebellum of mice in which the cerebellum had been bisected prior to injection. Another group of animals received the same injection into intact cerebella, i.e. without prior bisection. We found that bisection of the cerebella delayed the spread of scrapie agent from the injection site to the contralateral side of the cerebellum and that the occurrence of vacuolization was not as extensive and was markedly delayed in the un.injected side compared to its occurrence after injection in the intact cerebellum. Replication of agent in an area preceded the development of vacuolization in that area by several weeks. There was marked loss of Purkinje cells on the injected side of bisected cerebella, with no loss seen on the un.injected side. The incubation period of scrapie disease in mice injected after cerebellar bisection was significantly longer than after the injection of intact cerebella. The results in this study suggest that the scrapie agent spreads along intact nerve cell tracts, probably by axonal transport.

Key Words: Cerebellar bisection; Purkinje cell loss; Scrapie; Scrapie agent spread.

INTRODUCTION

Scrapie is the archetype of the unconventional slow infection diseases. The natural hosts for the scrapie agent are sheep and goats and the agent has been passaged experimentally in a variety of small animals, including mice and hamsters (1–4). Experimental systems using inbred strains of mice, well-characterized scrapie strains and different routes of injection have yielded important information about pathogenesis, pathology and genetics of the unconventional slow infections (5, 6).

A number of observations suggest that after injection, either into the central nervous system (CNS) or by non-CNS routes, the scrapie agent does not simultaneously invade all brain areas but rather spreads along nerve tracts (7, 8). For example, mice injected intraocularly initially showed asymmetrical vacuolization and infectivity in the contralateral superior colliculus. Vacuolization and infectivity occurred later in other regions of the brain including the ipsilateral superior colliculus. In other studies, intraperitoneal injection led to the sequential spread of agent from spleen to thoracic cord, then to lumbar and cervical cord and finally to the brain, with the initial appearance of infectivity in the posterior region of the brain and finally in the anterior region (9, 10).

One of the hallmark histopathological changes seen in scrapie is vacuolization and, although it is typically bilaterally distributed, after injection with certain scrapie strains lesions develop asymmetrically (11). Recently, we showed that the 22L scrapie strain injected directly in the cerebellum by the stereotactic method caused vacuolization only in the posterior regions of the brain, particularly the cerebellum,

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whereas other brain injection sites yielded vacuolization throughout the brain (12). These last two observations suggest that effects of the agent do not develop throughout the brain but appear in specific regions.

To assess the mode of spread of scrapie agent in the brain, scrapie agent was injected into one side of the cerebellum of mice in which the cerebellum had been bisected before injection; another group of animals received the same injection into the cerebellum without prior bisection. We found that bisection of the cerebellum delays the spread of scrapie agent from the injection site to the contralateral side and also delays or prevents the occurrence of pathological changes in the uninjected side. The onset of clinical disease occurred significantly later in the bisected group of mice compared to the group injected in intact cerebella.

**MATERIALS AND METHODS**

*Mice and Scrapie Strain:* Female weanling C57BL mice were obtained from Jackson Laboratories, Bar Harbor, ME, USA. Following injection at six to eight weeks of age, they were maintained in our animal colony with a 12 hour (h) on 12 h light cycle and were given food and water *ad libitum*. The 22L strain of scrapie agent was kindly provided by Dr. Alan Dickinson (Neuropathogenesis Unit of the MRC and AFRC, Edinburgh, Scotland) and was passaged serially by intracerebral injection of C57BL female mice. At the time of clinical disease, brains were removed under sterile conditions and 10% homogenates prepared in phosphate-buffered saline (PBS). All homogenates were stored at −70°C before use.

*Cerebellar Bisection and Stereotactic Injection Procedure:* Cerebellar bisection and microinjections were carried out under general anesthesia (sodium pentobarbital 50 mg/kg injected intraperitoneally). Anesthetized mice were mounted in a stereotactic apparatus (Stoelting, Chicago) at both ears and the nose. A longitudinal incision was made in the posterior scalp, and the occipital bone was exposed. Midline cutting of the occipital bone was done with a dental drill and bisection of cerebellum was done with the aid of the stereotactic apparatus and using a No. 11 surgical blade (3 mm depth, longitudinal cut through the midline). Histologically the cut was shown to separate completely the two sides of the cerebellum. The term “intact” cerebellum was used to refer to those instances in which the cerebellum was not bisected. Ten minutes after the surgery mice were stereotactically injected using a 30-gauge stainless steel needle; 2 µl of a 1% 22L scrapie brain homogenate was injected into the right side of the cerebellum. The coordinates used for cerebellum injection were A −6.5, L 1.5 and H +2.0 and for cerebral cortex injection the coordinates were A +1.0, L +2.0, H +1.5 (13).

*Clinical Evaluation:* Starting from ten weeks post-injection (pi), we examined all mice weekly for clinical symptoms. The clinical test consisted of monitoring motor coordination on a grid apparatus containing a series of parallel bars of 3 mm diameter, placed 7 mm apart from each other (14). An animal was scored positive when it failed to walk on the grid without foot slippage between bars. The incubation period was defined as ending on the third consecutive weekly positive score. Mice were killed for histological examination at different time intervals from six to 18 weeks pi except in those experiments in which survival times were monitored.

*Histopathological Evaluation:* Brains were removed and fixed by immersion in 10% neutral formalin. After fixation, brains were cut coronally at the cerebellum level. All tissues were paraffin-embedded and 7 µm sections were stained with hematoxylin and eosin (H&E). Each of the brain sections was given a score from 0 to 5 depending on the density of vacuolization and spongy state (15).

*Measurement of Scrapie Infectivity in Various Brain Regions:* Four, eight and 12 weeks pi brains from the cerebellar bisected and the intact groups were removed under aseptic conditions and sectioned as follows: Brains were cut into right and left cerebrum, and right and left cerebellum. Medulla was not used. A separate set of instruments was used to dissect each section in order to prevent cross-contamination. Homogenates (1%) were prepared from each section and then frozen at −70°C before titration. At the time of infectivity assay, homogenates were serially diluted using ten-fold dilutions in PBS. Mice used for assay were injected by
### TABLE 1
Incubation Periods and Survival Times of C57BL Mice Injected Stereotactically with Scrapie Strain 22L in Bisected, or Intact, Cerebella, or the Cerebral Cortex

<table>
<thead>
<tr>
<th>Injection site</th>
<th>Incubation period</th>
<th>Survival time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebral cortex</td>
<td>136 ± 1</td>
<td>152 ± 3</td>
</tr>
<tr>
<td>Bisected cerebellum</td>
<td>137 ± 2</td>
<td>153 ± 3</td>
</tr>
<tr>
<td>Intact cerebellum</td>
<td>112 ± 1†</td>
<td>137 ± 3*</td>
</tr>
</tbody>
</table>

Comparisons were between the group injected in the cerebral cortex and the groups injected in intact or bisected cerebella. *p < 0.01, †p < 0.001, (n = 15).

Routine intracerebral injection (not stereotactic) with 30 μl of the dilutions. Titers were calculated by the Reed-Muench formula (16).

**Statistical Analyses:** Data for incubation periods and survival times were evaluated using Student's *t*-test. Results exceeding the *p* < 0.05 level were regarded as significant.

### RESULTS

**Incubation Periods and Survival Times in Mice Injected Stereotactically in Cerebral Cortex and in Bisected and Intact Cerebella:** In results confirming previous findings (12), the average incubation period in mice injected stereotactically in intact cerebella with 22L was significantly shorter (*p < 0.001*) than that of mice injected in the cerebral cortex (Table 1); average survival time was also significantly shorter (*p < 0.01*) in the group injected in intact cerebella. Both incubation period and survival times for mice injected in bisected cerebella were significantly longer than for mice injected in intact cerebella (Table 1). The incubation period and survival time for mice injected in bisected cerebella and cerebral cortex were not significantly different.

**The Occurrence of Vacuolization in Mice Injected Stereotactically in Intact and Bisected Cerebella:** At the end of the incubation period, vacuolization was observed throughout the cerebellum in the group of mice injected in the intact cerebella, whereas in the bisected group vacuolization was limited to the side of the cerebellum that had been injected (Fig. 1). In order to assess the kinetics of the development of vacuolization, mice with bisected and intact cerebella were killed for histological examination at different times from six to 18 weeks pi. In the intact group of animals, vacuolization began ten weeks pi and increased throughout the rest of the incubation period (Fig. 2). The vacuolization pattern was symmetrical at all times. In the mice with bisected cerebella, vacuolization also began at ten weeks pi in the injected side and the intensity increased steadily throughout the rest of the incubation period. The rate of increase in vacuolization in the injected side was the same in intact and bisected cerebella. In the bisected group vacuolization was seen only in the side that had been injected from ten to 17 weeks pi. At 18 weeks pi there was extensive vacuolization in sections from the injected side and a few vacuoles were seen in the un.injected side. Vacuolization in forebrain regions was absent until 18 weeks in groups injected in either intact or bisected cerebella.

**Differential Effects on Purkinje Cells in Injected and Uninjected Sides of Cerebella:** In sections obtained from mice 16 weeks pi, there was an obvious reduction in the number of Purkinje cells on the injected side of the cerebellum compared to the uninjected side (Fig.3). In some areas on the injected side, there was almost complete loss of Purkinje cells; these areas were often close to sections that showed intense vacuolization. Some of the Purkinje cells that remained were swollen, whereas others
Fig. 1. Vacuolization pattern in cerebellum after cerebellar bisection, (a: uninjected side, b: injected side) in C57BL mice injected stereotactically in the right side of the cerebellum with 22L scrapie agent. Arrowheads indicate bisection cut. H&E. ×60.

appeared shrunken. The Purkinje cells on the uninjected side appeared to be normal at the light microscopy level with hematoxylin and eosin (H&E) stains. Loss of Purkinje cells on the injected side of the cerebellum was seen as early as 14 weeks pi; the loss at 14 weeks was much less pronounced than at subsequent times. Before 14 weeks pi there was no visible effect on Purkinje cells.

Scrapie Infectivity Titers in Different Brain Regions after Stereotactic Injection in Intact and Bisected Cerebella: It was reported previously that in mice injected stereotactically in the cerebellum with 22L, there were high titers of infectivity in the forebrain at 16 weeks pi despite the absence of vacuolization in that region (12). In fact, titers in the forebrain were similar to those in the cerebellum where there was extensive vacuolization. A possible explanation for the absence of vacuolization in the forebrain despite high levels of infectivity is that histopathological changes lag behind the spread and replication of agent. To test this explanation the levels of infectivity in different brain regions were assessed at four, eight and 12 weeks pi and the data compared to the intensity of vacuolization (Fig. 2) after stereotactic injection.

![Graph showing vacuolization scores](image)

Fig. 2. Vacuolization scores in the cerebellum (left panel-intact cerebellum group; right panel-bisected cerebellum group) of C57BL mice injected stereotactically with 22L scrapie agent in the right side of the cerebellum (vacuolation scores for the right side of the cerebellum •---•; vacuolation scores for the left side of the cerebellum •---•).
of intact and bisected cerebella. As shown in Table 2, infectivity levels in cerebrum homogenates tended to be lower than those found in cerebellum homogenates at each time point tested. This was demonstrated by assessing the proportion of animals positive or the incubation period (shorter incubation periods indicate a higher titer). Cerebellar infectivity was present long before vacuolization was first observed at ten weeks pi (Fig. 2), a situation similar to that noted for cerebrum in which high levels of infectivity were seen at 12 weeks pi (Table 2) but vacuolization was evident only at 18 weeks pi (12).

Comparison of scrapie infectivity levels in the right and left portions of the cerebellum showed that infectivity in the left side was found much earlier in mice injected in intact cerebella than in those with bisected cerebella (Table 2). An additional observation from the data in Table 2 is that at each time point infectivity levels tended to be higher in mice injected in intact cerebella compared to those injected in bisected cerebella. This was seen for homogenates prepared from both cerebellar hemispheres as well as from both cerebral hemispheres.

Previous studies showed that after stereotactic injection of intact cerebella the levels of infectivity at 19 weeks pi were similar in the four brain quadrants, the right and left cerebellum and the right and left cerebrum (12). After the injection of bisected cerebella, infectivity titers in cerebellum and cerebrum were higher at 19 weeks than at 12 weeks and the titers in different areas (e.g. cerebrum, right and left cerebellum) were similar (data not shown), indicating that the low titers in left cerebellum and forebrain areas through 12 weeks pi reflected a delay in agent replication in those areas.

**DISCUSSION**

Differences between mice injected stereotactically in intact versus bisected cerebella with the 22L scrapie strain all point to reduced spread of agent in bisected animals. The key findings include: 1) longer incubation period and survival time in mice injected in bisected cerebella; 2) a delay in the occurrence of pathological changes and a delay in high levels of infectivity in the uninjected side of bisected cerebella compared to the occurrence after injection of intact cerebella; 3) lower titers in the four brain regions tested in mice injected in bisected cerebella. It appears that if nerve tracts and neuronal connections are disrupted, there is a delay in spread of
TABLE 2
Distribution of Infectivity after Stereotactic Injection of Bisected or Intact Cerebella

<table>
<thead>
<tr>
<th>Time post injection</th>
<th>Brain area</th>
<th>%-Positive for scrapie</th>
<th>Incubation period</th>
<th>%-Positive for scrapie</th>
<th>Incubation period</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 weeks</td>
<td>Right cerebellum</td>
<td>100</td>
<td>175 ± 8*</td>
<td>100</td>
<td>162 ± 3</td>
</tr>
<tr>
<td></td>
<td>Left cerebellum</td>
<td>12</td>
<td>—</td>
<td>60</td>
<td>171 ± 3</td>
</tr>
<tr>
<td></td>
<td>Right cerebrum</td>
<td>12</td>
<td>—</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Left cerebrum</td>
<td>0</td>
<td>—</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>8 weeks</td>
<td>Right cerebellum</td>
<td>100</td>
<td>155 ± 3</td>
<td>100</td>
<td>143 ± 3</td>
</tr>
<tr>
<td></td>
<td>Left cerebellum</td>
<td>25</td>
<td>—</td>
<td>88</td>
<td>160 ± 3</td>
</tr>
<tr>
<td></td>
<td>Right cerebrum</td>
<td>25</td>
<td>—</td>
<td>37</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Left cerebrum</td>
<td>50</td>
<td>206 ± 0</td>
<td>25</td>
<td>—</td>
</tr>
<tr>
<td>12 weeks</td>
<td>Right cerebellum</td>
<td>100</td>
<td>141 ± 5</td>
<td>100</td>
<td>131 ± 3</td>
</tr>
<tr>
<td></td>
<td>Left cerebellum</td>
<td>100</td>
<td>142 ± 3</td>
<td>100</td>
<td>131 ± 3</td>
</tr>
<tr>
<td></td>
<td>Right cerebrum</td>
<td>50</td>
<td>155 ± 2</td>
<td>100</td>
<td>136 ± 3</td>
</tr>
<tr>
<td></td>
<td>Left cerebrum</td>
<td>62</td>
<td>149 ± 5</td>
<td>100</td>
<td>142 ± 2</td>
</tr>
</tbody>
</table>

* Incubation period in days (mean ± standard error). Incubation period means were not calculated if less than 50% of animals were positive for scrapie.

Infectivity. This supports the concept that scrapie agent spreads along nerve tracts. In a study using intracerebral injection, both vacuolization and infectivity occurred first in the contralateral superior colliculus and only much later were these observed in the ipsilateral superior colliculus (7, 8). Thus, the kinetics of infectivity and vacuolization were consistent with infectivity spreading in the optic nerve and crossing to the contralateral side at the optic chiasma. In a second study, the sequence of occurrence of infectivity after intraperitoneal injection was spleen, thoracic cord at vertebrae 6–9, and then areas caudal and rostral to that location (9, 10). The interpretation of these data was that agent spread from spleen along the splanchnic nerve to thoracic cord and thence to cervical and lumbar regions of the cord. After spread to the cervical cord, infectivity was found first in the posterior portion of the brain and then in the anterior portion. The concept of neural spread is supported further by the finding that direct injection into the sciatic nerve leads to a shorter incubation period than deposition of an equivalent dose of infectivity on the surface of the nerve (17).

The levels of infectivity in every region tested were higher in mice injected in intact cerebella than in those injected in bisected cerebella. In each instance, a likely explanation of this difference involves reduced spread of agent from the injection site to the brain region being assayed for infectivity. If intact nerve tracts are required for spread of infectivity, then disruption of these tracts would clearly lead to reduced titers on the uninjected side of the cerebellum. For the forebrain the lower titer following injection of bisected cerebella may reflect the reduced number of sites in the cerebellum that can act as centers for the spread of infectivity to the cerebrum. The surprising comparison was between the injection sides in intact versus bisected cerebella: At each time point, titers for the injected side of intact cerebella were higher than those for bisected cerebella. One possible explanation is that early after injection replication sites on the contralateral side of the cerebellum are not attainable because of the bisection, which in turn would reduce the quantity of infectivity that could “return” to the side that had been injected and establish new “centers” of

replication. A second explanation is that the Wallerian degeneration that follows the bisection of nerve tracts destroys nerve cells that could serve as replication centers for the agent. Finally, it is possible that the influx of fluid or the cells of the reticuloendothelial system at the site of bisection might reduce the effective infectivity of the inoculum and cause the reduced titers noted in Table 2. It seems more likely, however, that the disruption of nerve tracts and the consequent reduction in infected "centers" led to the lower titers in the injected side of bisected cerebella.

The loss of Purkinje cells in experimental scrapie has not been reported before although effects have been described in naturally occurring scrapie in sheep (18). The marked difference in effect between injected and non-injected sides of the cerebellum made this feature easy to assess. It would be interesting to examine sections from mice injected with 22L in intact cerebella and those injected by other routes. In addition, it would be important to examine other scrapie strain-mouse strain combinations for effects on Purkinje cells; these studies would allow the assessment of the role of route of injection, mouse genetics and scrapie strain.

The findings in this report support the concept that in the central nervous system scrapie spreads along intact nerve tracts. The report includes additional evidence showing that replication of agent in an area of the brain precedes the development of vacuolization in that area by several weeks.

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