Incubation Periods and Survival Times for Mice Injected Stereotaxically with Three Scrapie Strains in Different Brain Regions

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SUMMARY

Incubation period and survival time were determined in C57BL mice which had been injected stereotaxically with either the 139A, ME7 or 22L strain of scrapie in one of five different brain regions (cerebral cortex, caudate nucleus, thalamus, substantia nigra, cerebellum). The injection of 139A in the caudate nucleus, thalamus, substantia nigra or cerebellum resulted in significantly shorter incubation periods than following cerebral cortex injection. For ME7, mice injected in the thalamus and cerebellum had incubation periods approximately 2 weeks shorter than the cerebral cortex group. For 22L, the incubation periods after substantia nigra or cerebellum injection were significantly shorter than after cortex injection. The cerebellum injection group had a significantly shorter incubation period than the substantia nigra injection group. The survival times for mice injected with a particular scrapie strain were directly related to the incubation periods. The groups with the shortest incubation also had the shortest survival time (e.g. 22L in the cerebellum). On histological examination, 139A and ME7 produced brain lesions in all brain areas regardless of injection site. For the 22L strain, after cerebellum injection vacuolation was limited to the cerebellum, while injection into the cerebral cortex and other forebrain regions resulted in vacuolation in all brain regions examined. Despite the difference in the distribution of vacuolation seen in cerebellum- compared to cortex-injected (22L) mice, infectivity titres were similar in the cortex, cerebellum, cerebellar cortex and medulla plus pons.

INTRODUCTION

Scrapie agent causes a slow degenerative neurological disease in sheep and goats (Hadlow et al., 1979) and is similar to the transmissible agents causing certain dementias in humans such as Kuru, Creutzfeldt-Jakob disease and Gerstmann-Straussler syndrome (Gajdusek, 1977; Masters et al., 1981).

During the past 20 years, a variety of experimental scrapie models have been developed in mice (Dickinson et al., 1984; Kimberlin, 1984). These models differ in a broad range of biological and histopathological parameters. The differences are controlled by both host and scrapie strain. For example, incubation period is in part dependent on Sinc, which is the major mouse gene that controls scrapie incubation (Dickinson & Fraser, 1979), and in part by the scrapie strain (Dickinson et al., 1984; Carp et al., 1985, Carp & Callahan, 1986). In addition, the distribution and intensity of vacuolation differs depending on both scrapie strain and the inbred mouse strain used (Fraser, 1976). The principal type of lesion is vacuolar degeneration, typically with a bilateral, symmetrical distribution (Fraser, 1979). However, asymmetrical lesions can occur in the brains of mice inoculated intracerebrally or by peripheral routes using certain strains of scrapie (87A, 31A, 125A) (Bruce & Fraser, 1982). In addition, mice inoculated intraocularly with the ME7 strain initially show asymmetrical vacuolation of the contralateral colliculus (Fraser, 1982).

Two studies have reported functional changes indicative of selective effects in the brain.
one, progressive destruction of the striatal neurons on the side of stereotaxic injection of scrapie strain 263K into the hamster substantia nigra was demonstrated by the circling behaviour of the hamster following apomorphine injection (Gorde et al., 1982). Recently, Carp et al. (1984) showed that some strains of scrapie could induce increases in body weight during the preclinical phase of disease in mice whereas other strains had no effect. It was suggested that the differences in scrapie strain effects were dependent on agent effects on the hypothalamus.

Scrapie agent given intraperitoneally (i.p.) started to replicate at different times in different regions of the central nervous system (CNS) (Kimberlin & Walker, 1982). These observations suggest that scrapie does not simultaneously invade all CNS structures. In subsequent work, it was shown that the duration of replication in brain was longer after intracerebral (i.c.) injection than after an i.p. injection (Kimberlin & Walker, 1983, 1986). This led the authors to postulate that the development of scrapie depends on agent spreading to and replicating in certain areas in the brain termed clinical target areas (CTA), and that agent entering the CNS in the thoracic cord, after peripheral infection, is transported more directly to these target areas than agent injected i.c. in the frontal lobe. In this context the CTA concept has two components: (i) there are cells controlling the development of clinical disease that are vulnerable to scrapie effects and (ii) the agent must spread from the point of injection to these cells. In the present study, we attempted to determine the CTA for three scrapie strains. We bypassed the step involving agent spread to CTA by injection directly into five different brain regions. The three scrapie strains were injected stereotaxically into the cerebral cortex, caudate nucleus, thalamus, substantia nigra or cerebellum, and incubation period, vacuolation and, in some instances, survival time were measured.

**METHODS**

**Mice.** Female weanling C57BL mice were obtained from Jackson Laboratories, Bar Harbor, Me., U.S.A. Following injection at 6 weeks of age, they were maintained in our animal colony with a 12 h on-12 h off light cycle and were given food and water *ad libitum.*

**Scrapie strains.** Three strains of scrapie were used: ME7 and 22L, provided by Dr Alan Dickinson (Edinburgh, U.K.), and 139A by Dr Richard Kimberlin (Edinburgh). These strains were passaged in our laboratory using C57BL mice. Passages were done by i.c. injection of 30 μl of 1% brain homogenate (uncentrifuged) prepared from mice clinically ill with scrapie. Brains were stored frozen at −70 °C prior to homogenization. All brain homogenates were prepared in cold (4 °C) phosphate-buffered saline (PBS) containing CaCl₂ (0.01%) and MgCl₂ (0.005%). Homogenization was done using 20 strokes in a hand-operated Ten-Broek homogenizer.

Mice injected with normal mouse brain served as a source for normal brain homogenate and these homogenates were prepared as described for scrapie preparations.

**Stereotaxic injection.** Microinjections were carried out under general anaesthesia (sodium pentobarbital 70 mg/kg i.p.) using a stereotaxic instrument (Stoelting Co.). A total of 288 mice were injected in either the cerebral cortex, caudate nucleus, thalamus, substantia nigra or cerebellum (96 for each scrapie strain) with one of three scrapie strains, ME7, 22L or 139A. For each scrapie strain, the cerebral cortex, thalamus and cerebellum groups consisted of 24 mice each, and the other two groups 12 mice. Another 72 mice were injected either with 22L or ME7 in the cerebral cortex, thalamus or cerebellum (12 per group). These animals were observed until death to measure the survival time as well as incubation period.

Since no cerebral dominance for scrapie infection has been shown, injections were made in the right hemisphere.

The stereotaxic coordinates used for the five brain regions were as follows: the cerebral cortex, A +1.0, L +2.0, H +1.5; caudate nucleus, A +0.5, L +2.0, H +3.5; thalamus, A −1.4, L +0.8, H +3.2; substantia nigra, A −3.4, L +1.4, H +4.0; cerebellum, A −6.5, L +1.0, H +2.0 (Slotnik & Leonard, 1975). Using a 30-gauge stainless steel needle, 5 μl of a 1% scrapie brain homogenate was injected into an appropriate brain region. In order to minimize pressure-induced spread of inoculum, 1 to 2 min were taken for each stereotaxic injection. In preliminary experiments similar volumes of normal mouse brain homogenate containing carbon particles were injected into the five regions. Brains from these animals were examined for the distribution of carbon particles and their location was consistent with the region specified by the coordinates. Particles were not observed in regions surrounding the target nor in the regions lying in the path of the needle. Injection of normal mouse brain homogenates was done using the same procedures as those used with scrapie material.

**Clinical evaluation.** Starting from 10 weeks post-injection, all mice were examined 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 (Carp et al., 1984). An animal was scored positive when it
failed to walk on the grid without foot slippage between bars. In positive mice this initial sign was followed by increasing locomotor difficulties, with eventual weakening, wasting and death. Mice were observed on the grid for at least 20 s and, in those instances in which there were questionable findings, the length of observation could reach several minutes. The reliability of the test was supported by the fact that it was rare that a positive score was reversed on subsequent evaluations. The incubation period was defined as ending on the third consecutive weekly positive score. Mice were sacrificed within 1 week of the end of the incubation period except in those experiments in which survival times were monitored (Table 2).

**Histopathological evaluation.** Brains were removed and fixed by immersion in 10% neutral formalin. After fixation, brains were cut coronally at four standard levels (Fraser & Dickinson, 1968). All tissues were paraffin-embedded and 7 μm sections were stained with haematoxylin and eosin. Nine different regions of the grey matter were examined (without knowledge of the sample origin) under the light microscope: (1) dorsal medulla, (2) cerebellar cortex, (3) mesencephalon, (4) hypothalamus, (5) thalamus, (6) hippocampus, (7) paraterminal body, (8) posterior cerebral cortex and (9) anterior cerebral cortex. Each of the nine areas was given a score from 0 to 5 depending on the density of vacuolation and status spongiosus (Fraser & Dickinson, 1968; Fraser, 1976).

**Measurement of infectivity in different brain regions.** Mouse brains were removed under aseptic conditions and sectioned as follows. One hemisphere was cut into cerebrum and cerebellum whereas the other hemisphere was divided into cerebrum, cerebellar cortex and medulla plus pons. A separate set of instruments was used to dissect each section in order to prevent cross-contamination. Homogenates (1%) were prepared from each section as described previously (see paragraph on scrapie strains) and then frozen at −70 °C prior to titration. Homogenates were serially diluted through a series of ten-fold dilutions in PBS. Mice were injected by routine i.c. injection (not stereotaxic) with 30 μl of the dilutions. Titres were calculated by the Reed–Muench formula (1938).

**Statistical analyses.** The data for evaluating clinical incubation period were analysed by analysis of variance with scrapie strain and injection site as the main effects. Individual means were compared by multiple comparison tests. Results exceeding the P < 0.05 level were regarded as significant.

**RESULTS**

**Incubation periods in mice injected stereotaxically with three scrapie strains in five brain regions**

Incubation periods in mice receiving one of three strains of scrapie in different brain regions are presented in Table 1. Stereotaxic injection of 139A strain in the caudate nucleus, thalamus, substantia nigra and cerebellum resulted in significantly shorter incubation periods, by 20 days or more, than did cerebral cortex injection. Unlike 139A, injection with ME7 strain in the caudate nucleus and substantia nigra did not yield significantly different incubation periods from the cerebral cortex. However, mice injected in the thalamus and cerebellum had incubation periods approximately 2 weeks shorter than the cerebral cortex group. For 22L-injected mice, the cerebellum and substantia nigra injection groups had shorter incubation periods (statistically significant) than the cerebral cortex group. The incubation period for the cerebellum injection group was significantly shorter than that for the substantia nigra injection group (113 days compared to 126 days). In the group of mice injected in the cerebellum with 22L, ataxia developed approximately 16 days prior to the appearance of any of the other typical scrapie signs. During these 16 days, the ability of mice to stand and walk on the parallel bars used for scoring was not impaired and their only clinical abnormality was ataxia. In other groups, ataxia, which is a characteristic scrapie symptom, began at the same time or after the mice showed locomotion difficulty on the bars.

**Survival time following stereotaxic injection**

Incubation periods of additional groups of mice receiving 22L or ME7 in three brain regions (Table 2) were almost identical to the values shown in Table 1. For each scrapie strain, survival time was directly related to the incubation period, that is, for 22L both incubation and survival time were significantly shorter for the cerebellum injection group than for the cerebral cortex and thalamus groups. For ME7, both thalamus and cerebellum injections led to shortened incubation periods as well as survival times as compared to the cortex injection group.

**Neuropathological findings**

Histopathological examination was done on those mice receiving stereotaxic injections of scrapie agent in the cerebral cortex, thalamus or cerebellum. Mice were killed within 1 week of
Table 1. Incubation periods of scrapie in C57BL mice injected in different brain regions

<table>
<thead>
<tr>
<th>Injection site</th>
<th>Scrapie strain</th>
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<tbody>
<tr>
<td></td>
<td>139A</td>
</tr>
<tr>
<td>Cerebral cortex (n = 24/group)</td>
<td>153 ± 4*</td>
</tr>
<tr>
<td>Caudate nucleus (n = 12/group)</td>
<td>132 ± 1†††</td>
</tr>
<tr>
<td>Thalamus (n = 24/group)</td>
<td>133 ± 2††</td>
</tr>
<tr>
<td>Substantia nigra (n = 12/group)</td>
<td>131 ± 2†††</td>
</tr>
<tr>
<td>Cerebellum (n = 24/group)</td>
<td>130 ± 1†††</td>
</tr>
</tbody>
</table>

* Incubation period in days (mean ± standard error).
† Third positive score for ataxia.
††† For each inoculum, comparisons were between groups injected in each region and those injected stereotaxically in the cerebral cortex. †P < 0.05, ††P < 0.01, †††P < 0.001.

Table 2. Relationship of incubation period and survival time following stereotaxic injection of scrapie in C57BL mice injected in different brain regions

<table>
<thead>
<tr>
<th>Injection site</th>
<th>Scapie strain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>22L</td>
</tr>
<tr>
<td>Cerebral cortex (n = 12/group)</td>
<td>138 ± 2*</td>
</tr>
<tr>
<td>Thalamus (n = 12/group)</td>
<td>135 ± 2</td>
</tr>
<tr>
<td>Cerebellum (n = 12/group)</td>
<td>118 ± 1†††</td>
</tr>
</tbody>
</table>

* Number of days (mean ± standard error).
††† For each inoculum, comparisons were between groups injected in each region and those injected stereotaxically in the cerebral cortex. ††P < 0.01, †††P < 0.001.

the end of the incubation period. Mice injected with normal brain homogenate were killed at a time after injection equivalent to that for mice injected in the same region with scrapie. The lesion scores of the 139A group are depicted in Fig. 1, ME7 in Fig. 2, and 22L in Fig. 3. For 139A and ME7, the extent of vacuolation in the nine different brain regions showed the same pattern, regardless of the injection site. For 22L, vacuolation patterns for the cerebral cortex and thalamus groups were similar to those reported for routine i.c. injections (Fraser, 1979); however, the cerebellar injected group had a high lesion score only in the cerebellum, with little or no vacuolation found in other areas.

Infectivity titres in different brain regions

Differences in the vacuolation pattern after cortex and cerebellum injections of 22L prompted an analysis of infectivity titres in these mice. Brain sections obtained from two mice which had been injected stereotaxically with 22L in the cortex or in the cerebellum were analysed for infectivity. Mice were sacrificed within 1 week after their being scored positive for the third consecutive week. The sections included cerebrum and cerebellum from the right side and the cerebrum, cerebellar cortex and medulla plus pons from the left side. Titre differences among the various sections were minimal for both cortex- and cerebellum-injected mice, with no more than 0.6 log10 unit difference between any two sections from the same mouse. For the two mice injected in the cortex, the titres per gram of tissue ranged from 10^8.4 to 10^9.9 LD50 for one mouse and 10^8.2 to 10^8.3 LD50 for the other mouse. For the mice injected in the cerebellum, the titres
Clinical course of scrapie in mice

Fig. 1. Lesion profiles (vacuolation) in the grey matter of mice injected with 139A scrapie in the cerebral cortex (□), thalamus (●) or cerebellum (○). Positions 1 to 9 are (1) dorsal medulla, (2) cerebellar cortex, (3) mesencephalon, (4) hypothalamus, (5) thalamus, (6) hippocampus, (7) paraterminal body, (8) posterior cerebral cortex and (9) anterior cerebral cortex. A score of 5 represents the highest density of lesions and a score of 0 no lesions. Each point is the mean value from five to 11 mice and standard errors were < ±0.3. The mean vacuolation score for age-matched controls was ≤0.35.

Fig. 2. Lesion profiles in the grey matter of mice injected with ME7 scrapie in the cerebral cortex (□), thalamus (●) or cerebellum (○). For position designations, see Fig. 1. Each point is the value from seven to 14 mice and standard errors were < ±0.4. The mean vacuolation score in age-matched controls was ≤0.25.

Ranges from 10^{8.0} to 10^{8.6} LD_{50} for one mouse and from 10^{8.0} to 10^{8.3} LD_{50} for the other mouse. Furthermore, even differences between two sections were not consistent in samples from the two mice, e.g. in one mouse injected i.c. the cortex had a titre of 10^{8.0} LD_{50}/gram and the cerebellum 10^{8.6} LD_{50}/gram whereas in the second mouse the titres were 10^{8.2} LD_{50}/gram in both sections.

DISCUSSION

The key findings of the present study show that injection in different brain regions can at the very least affect incubation period, lesion distribution and survival time. The data support the concept of CTA (Kimberlin & Walker, 1983, 1986). Thus, there are regions in which scrapie
Fig. 3. Lesion profiles in the grey matter of mice injected with 22L scrapie in the cerebral cortex (□), thalamus (○) or cerebellum (●). For position designations, see Fig. 1. Each point is the value from seven to 13 mice and standard errors were ≤ ± 0.3. The mean vacuolation score in age-matched controls was ≤ 0.35. The mean vacuolation scores in positions 4 to 9 of the 22L cerebellum injection group were ≤ 0.4.

agent causes the cellular effects that lead to clinical disease more quickly than it does in other regions. This implies that replication and the induction of lesions in much of the CNS may be irrelevant for disease. The concept of CTA arose from the observation that the duration of replication in the brain is always longer after i.c. injection than after non-neural, peripheral injection (Kimberlin & Walker, 1983, 1986). The data suggested that the spread of agent along peripheral nerves to the spinal cord after i.p. injection leads to infection of the clinical target areas (probably located in the brain stem or cerebellum) more quickly than after i.c. injection.

In the current study, results with the three scrapie strains differed. For 22L, mice injected in the cerebellum had a significantly shorter incubation period than mice injected in any other region. These findings suggest that the cerebellum or a region(s) near it is most susceptible to the induction of clinical disease by 22L scrapie agent. The selective vulnerability of the cerebellum to 22L-induced vacuolation was suggested by Fraser (1979) who found that following either i.c. or i.p. injection of 22L, extensive vacuolation was seen in the cerebellum, more than with any other scrapie strain. In the present study, mice injected stereotaxically with 22L in the cortex, caudate nucleus, substantia nigra or thalamus all showed a distribution of vacuolation similar to that seen with routine (non-stereotaxic) i.c. injection, including extensive vacuolation in the cerebellum. The results with cerebellar injection of 22L were remarkable in that vacuolation was limited to the cerebellum. These mice also developed a distinctive ataxic gait prior to the appearance of the typical motor incoordination used to score for scrapie disease. This early occurrence of ataxia was seen only with this combination of scrapie strain and route.

These clinical and pathological findings are similar to an unusual slow virus-induced neurological disease in humans, Kuru. Kuru, described in the Fore natives of New Guinea, was the first slow infection documented in human beings (Gajdusek, 1977). The disease begins insidiously with unsteadiness of stance and gait. A progressive symmetrical ataxia develops over a period of months. Pathological changes are confined to the brain where a marked increase of astrocytes and degeneration of neurons with cytoplasmic vacuolation are found. The brain is diffusely affected, but the findings are most prominent in the cerebellum and pons and, to a lesser degree, in the hypothalamus and basal ganglia (Gajdusek, 1977). Similarities between Kuru and scrapie were first noted by Hadlow (1959) on the basis of pathological and epidemiological findings. The results noted above for C57BL mice injected in the cerebellum with 22L establish this system as an ideal model of Kuru.
The results with ME7 also support the concept of CTA in that two areas, the cerebellum and thalamus, yielded markedly shorter incubation periods than injection of other regions. The 139A data revealed differences in incubation period according to the site of injection but failed to reveal a region that was particularly sensitive to the induction of clinical disease. Of course, this could just mean that a brain region not yet tested is the site for producing clinical disease with 139A. Similarly, a region different from those tested might give even shorter incubation periods for ME7 and 22L. It is possible that injection sites giving shorter incubation periods are not the most vulnerable to induction of clinical disease, but contain cells with projections into the most vulnerable sites.

The data on survival times support the incubation period data in that for each scrapie strain the relationships among the various groups were similar for the two parameters. This is important because incubation period data are dependent on the criteria used to assess initial clinical manifestations; in our case the methodology is most sensitive to motor dysfunction. Survival times would reflect damage to a life support system.

In mice injected with 22L in the cerebellum there were high levels of infectivity in the forebrain despite an absence of vacuolation. In previous studies a relationship between vacuolation and infectivity has not been clearly established. Some have argued that the more severe the brain vacuolation the higher the concentration of infectivity (Dickinson et al., 1978). Others, however, have presented evidence showing high concentrations of infectivity in brain regions which had no vacuolation (Hadlow et al., 1974, 1980). Marsh et al. (1976) have described a model of transmissible mink encephalopathy, a disease related to scrapie, in which clinically affected animals had high titres of agent in the brain but no discernible vacuolation. The most plausible explanation for the discrepancy may lie in a difference in timing of replication and vacuolation. According to Cole & Kimberlin (1985), 139A injected i.p. in Compton White mice began to show vacuolation in the brain stem which gradually spread to the forebrain regions. Prior to the appearance of vacuolation in the anterior brain these regions contained high levels of infectivity. In the present study, it is probable that agent replication occurred first in the cerebellum and led to pathological changes. Subsequent spread to the forebrain was followed by replication of agent in that area but signs and symptoms developed before vacuolation could occur (Cole & Kimberlin, 1985). A time course study following stereotaxic injection of 22L in the cerebellum has been initiated recently to test this putative sequence of events.

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