The Pathogenicity of the A7, M9 and L10 Strains of Semliki Forest Virus for Weanling Mice and Primary Mouse Brain Cell Cultures

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SUMMARY

The multiplication of the M9, A7 and L10 strains of Semliki Forest virus (SFV), both in weanling mice and primary mouse brain cell cultures, was compared. Following both intraperitoneal (i.p.) and intracerebral (i.c.) injection, the virulent L10 strain multiplied to higher titre in the mouse central nervous system (CNS) than did the less virulent M9 and A7 strains, whereas M9 multiplied to higher titre than A7. By the i.c. route, all three virus strains multiplied to higher titre than following i.p. injection. Multiplication of A7 and M9 in oligodendrocytes, but not neurons, was detected following i.c. injection. All three virus strains showed a tropism for cultured mouse glial cells rather than neurons. The L10 strain multiplied better in neurons than did A7 or M9. It is concluded that the mechanism of acute demyelination induced by the M9 and A7 strains is similar. Based on this and previous studies, it is proposed that infection of glial cells triggers immune-mediated demyelination. The virulence of the L10 strain is due to its ability to exceed a lethal threshold of damage to neurons before immune intervention can occur.

INTRODUCTION

Semliki Forest virus (SFV) infection of mice has been extensively used as a model to study the mechanisms of neurovirulence and virus-induced demyelination of the central nervous system (CNS; Atkins et al., 1985). Our recent studies have indicated that the virulence of the L10 strain is due to its ability to damage neurons, that the acute demyelination produced by the M9 mutant of SFV is triggered by a lytic infection of oligodendrocytes, and that demyelination is partially immune-mediated (Barrett et al., 1980; Atkins & Sheahan, 1982; Sheahan et al., 1983; Gates et al., 1984).

Other workers have studied demyelination by the avirulent A7 strain of SFV. Although initial studies indicated that demyelination was due to the cytolytic effect of the virus (Chew-Lim et al., 1977; Chew-Lim, 1979), it has subsequently been suggested that it is immune-mediated (Jagelman et al., 1978; Berger, 1980). However, the A7 strain was not detected in the brain by electron microscopy so that its site of multiplication was unknown (Pathak & Webb, 1978). We have shown that the avirulent A7 strain has similar molecular and host range properties to the neurovirulence mutant M9 (Atkins, 1983). Also, Bruce et al. (1984) have shown that infection of rat mixed glial cultures with A7 results in depletion of oligodendrocytes and fibrous astrocytes. Protoplasmic astrocytes and neurons (M. Rumsby, personal communication) remained intact. These results provided initial evidence that the mechanism of demyelination by A7 and M9 may be similar. In this study, we present further evidence from animal infection experiments and from infection of primary mouse neuron and glial cell cultures that the mechanism of acute demyelination by M9 and A7 is similar.
METHODS

General. The inbred BALB/c mice used in this study were originally obtained from Olac (1976) Ltd, Bicester, U.K. Breeding colonies were housed separately from infected animals, which were housed in a containment facility. Titration of infectious virus in blood and brain homogenates by plaque assay was carried out as described previously (Barrett et al., 1980; Sheahan et al., 1981). Antiserum to glial fibrillary associated protein (an astrocyte marker), and to galactocerebroside (an oligodendrocyte marker), were a gift from Dr M. Rumsby, University of York, York, U.K. Anti-A2B5 (a neuron marker) was a gift from Dr C. Reagan, University College, Dublin, Ireland. In preparing mouse neuron and mixed glial cell cultures, we adapted methods designed for the preparation of these cells from rat brain, as described below.

Multiplication of SFV strains in the mouse. Groups of 39- to 41-day-old mice were infected with 10⁴ p.f.u. of the A7, M9 or L10 strains of SFV given in 0.5 ml phosphate-buffered saline (PBS) for the intraperitoneal (i.p.) route, or in 0.02 ml PBS for the intracerebral (i.c.) route. In order to minimize the variation in brain titres following i.p. infection (Atkins & Sheahan, 1982), groups of five mice were sacrificed at 4 h, 12 h and daily intervals up to 14 days post-infection. Brain [10% (w/v) clarified homogenate] and blood [10% (v/v) suspension] extracts were pooled separately, and frozen at -70 °C before plaque assay. Preliminary experiments showed that brain titres following i.c. infection with SFV were less variable than those following i.p. infection. For i.c. infection, groups of three mice were sacrificed daily and brain extracts pooled. Additional groups of mice were injected to measure mortality only, over a 14 day period.

Pathology. A total of 23 39- to 41-day-old mice infected i.c. with either M9 or A7 were selected for examination. M9-infected mice were processed at 2 days (three mice), 5 days (three mice) and 6 days (three mice). A7-infected mice were processed at 5 days (three mice), 6 days (three mice), 7 days (five mice) and 14 days (three mice). The mice were anaesthetized with ether and perfused though the left ventricle with 0.02 ml PBS for the intracerebral (i.c.) route. In order to minimize the variation in brain titres following i.p. infection (Atkins & Sheahan, 1982), groups of five mice were sacrificed at 4 h, 12 h and daily intervals up to 14 days post-infection. Brain [10% (w/v) clarified homogenate] and blood [10% (v/v) suspension] extracts were pooled separately, and frozen at -70 °C before plaque assay. Preliminary experiments showed that brain titres following i.c. infection with SFV were less variable than those following i.p. infection. For i.c. infection, groups of three mice were sacrificed daily and brain extracts pooled. Additional groups of mice were injected to measure mortality only, over a 14 day period.

Preparation of neuron cultures. This was based on the method described by Wilkin et al. (1976). The cerebella were removed from 8-day-old mice and blotted onto sterile filter paper to remove the meninges. Groups of four cerebella were chopped finely in two directions at 90°. The pieces of tissue were transferred to a 50 ml conical tube containing 10 ml of Krebs–Ringer bicarbonate solution [KRB; 1.2 mM-sodium chloride, 48 mM-potassium chloride, 12.2 mM-potassium dihydrogen phosphate, 0.26 mM-sodium dihydrogen phosphate, 0.26 mM-sodium bicarbonate, 0.143 mM-glucose, 0.01% (w/v) phenol red] containing 0.3% (w/v) bovine serum albumin (KRB/BSA). On completion of the dissection the tube was centrifuged at 1000 r.p.m. for 30 s. The pellet was resuspended in KRB/BSA containing 0.25 mg/ml bovine pancreatic trypsin (type III, Sigma), rapidly transferred to a capped conical flask and shaken at 37 ºC for 5 to 8 min. The contents of the flask were then tipped into an equal volume of KRB/BSA containing 4 μg/ml DNase I (Sigma) and centrifuged at 1000 r.p.m. for 5 min. The supernatant was aspirated, and 2 ml of KRB/BSA containing 0.1 mM-calcium chloride added. The shearing cycle was repeated once more, the supernatants pooled, and the cells sedimented at 1000 r.p.m. for 5 min. The cells were resuspended in 10 ml of culture medium (Dulbecco’s modified MEM containing 10% calf serum, 25 mM-potassium chloride and 10 μg/ml gentamicin). Viable cells were plated at a density of 2.5 × 10⁶ in 2 ml of culture medium in 35 mm plastic dishes which had been pre-coated with poly-L-lysine (Sigma; dishes were treated with 1 ml containing 5 μg/ml for 10 min and the solution was removed). Proliferation of non-neuronal cells was prevented by the addition of cytosine arabinoside (Sigma) to a final concentration of 10 μM at 18 to 20 h after the cells were plated. The medium was changed at 3 days after plating, and the cells infected at 6 days after plating.

Preparation of glial cell cultures. This was based on the method described by Chapman & Rumsby (1982). Cerebra from 2-day-old neonatal mice were removed into culture medium (Dulbecco’s MEM supplemented with 10% foetal calf serum, 100 units/ml penicillin and 75-5 μg/ml streptomycin). Individual cerebra were divided along the longitudinal fissure into separate hemispheres and the meninges removed by blotting on sterile filter paper. Three cerebra were then placed in the barrel of a 1 ml disposable syringe. One ml of culture medium was added to the tissue, which was then expelled five times through a 19-gauge needle and ten times each through 21- and 23-gauge needles. The cells were then plated at a density of 7 × 10⁵ to 9 × 10⁵ cells per 35 mm plastic dish. The cells were incubated at 37 ºC in a 5% CO₂ atmosphere for 6 days and the medium changed every 3 days thereafter. Virus infection was carried out at 12 days after plating.

Infection of mouse brain cell cultures. Before infection, cells were removed from duplicate plates with trypsin–EDTA. The cells were then counted and discarded, and virus added to similar plates to give 100 p.f.u./cell. After adsorption for 1 h, the cells were washed twice with PBS, and medium was added. Infections were carried out in duplicate, and average results from two separate experiments are quoted. For viral RNA synthesis, the cells were pulse-labelled with 10 μCi/ml [3H]uridine in 2 ml of medium and in the presence of 5 μg/ml actinomycin D from 2
Table 1. Multiplication of SFV strains in the mouse*

<table>
<thead>
<tr>
<th>Route of Virus inoculation</th>
<th>Maximum virus content of blood (p.f.u./ml) (days post-infection)</th>
<th>Maximum virus content of brain (p.f.u./g) (days post-infection)</th>
<th>Mean time of death</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Titre (days post-infection)</td>
<td>Time reached</td>
<td>Mortality† (+ standard error)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L10</td>
<td>4 x 10⁵</td>
<td>2</td>
<td>3 x 10⁹</td>
</tr>
<tr>
<td>i.p.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i.c.</td>
<td>ND</td>
<td>ND</td>
<td>6 x 10¹⁰</td>
</tr>
<tr>
<td>M9</td>
<td>3 x 10⁵</td>
<td>2</td>
<td>1 x 10⁷</td>
</tr>
<tr>
<td>i.p.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i.c.</td>
<td>ND</td>
<td>ND</td>
<td>5 x 10⁹</td>
</tr>
<tr>
<td>A7</td>
<td>8 x 10⁶</td>
<td>1</td>
<td>8 x 10⁵</td>
</tr>
<tr>
<td>i.p.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i.c.</td>
<td>ND</td>
<td>ND</td>
<td>9 x 10¹⁰</td>
</tr>
</tbody>
</table>

* Mice were infected with 10⁴ p.f.u. Blood and brain samples were taken at 14 h, 12 h and daily intervals up to 14 days post-infection. Samples were pooled from five mice for i.p. injection and from three mice for i.c. injection.† Out of total number injected. ND, Not done, NA, not applicable.

to 10 h after infection, and the TCA-precipitable radioactivity was measured as previously described (Atkins & Sheahan, 1982). Protein synthesis was measured by pulse-labelling with [³⁵S]methionine 4 to 6 h after infection, and chasing for 1 h, as described by Atkins & Sheahan (1982). Virus yield was measured from culture fluids taken at 16 h after infection. At this time, the c.p.e. in the cultures was observed, fresh medium added, and the cultures were incubated further.

RESULTS

Multiplication of SFV strains in the mouse

The titres of virus recorded in blood and brain extracts following i.p. and i.c. infection are shown in Table 1. For the virulent L10 strain, all mice injected by either route died. For M9, the majority of mice infected i.p. survived, whereas the majority infected i.c. died. For A7, most mice injected by either route survived. Maximum viraemia produced by L10 and M9 was at 2 days after infection, similar in titre for both strains. For A7, viraemia was maximum at 1 day and was higher in titre than either L10 or M9. Virus was cleared from the blood at 5 days for A7, but at 4 days post-injection for M9. No clearance of viraemia occurred for L10, and maximum brain titres preceded death. For A7 and M9, brain titres were maximum at 6 days following i.p. injection, and cleared at 10 days post-infection. However, the maximum brain titre reached by A7 after i.p. infection was less than one-tenth of that reached by M9. Following i.c. injection, M9 again reached higher titre than A7, but the titre reached by both viruses was about 100 times that reached after i.p. infection.

Pathology

The lesions produced in the CNS following i.p. infection with M9 have been described previously (Atkins & Sheahan, 1982; Sheahan et al., 1983). We have confirmed the finding of Pathak & Webb (1978) that virus particles could not be detected by electron microscopy in the CNS of mice infected i.p. with A7, although lesions were apparent.

For mice infected i.c. with M9, the only lesions seen 2 days after infection were occasional mononuclear leukocytes in the leptomeninges of the brain and spinal cord. Mononuclear leukocytes were prominent in perivascular spaces at 5 days post-infection when focal areas of spongy change were randomly distributed in white matter at all levels. Lesions were common in the periventricular white matter of the cerebellum and at the exit zone of spinal nerve roots. Small cells with dense pycnotic nuclei were present in areas of spongy change, in otherwise normal white matter and adjoining neurons. Some of these cells were identified ultrastructurally as oligodendrocytes and contained intermediate stages of virus multiplication in the cytoplasm. Hypertrophic and vacuolated oligodendrocytes containing nucleocapsids and occasional virus particles, similar to those described following i.p. inoculation of the virus (Atkins & Sheahan, 1982; Sheahan et al., 1983), were also present. Spongy change was uniformly severe 6 days after...
Fig. 1. Virus-containing oligodendrocytes in the CNS of mice infected i.c. with A7 SFV. (a) Spinal cord at 7 days. An oligodendrocyte with intracytoplasmic vacuoles, membranous profiles and virus nucleocapsids is shown. Note the connection between the plasma membrane of the oligodendrocyte and the myelin sheath (arrow). Bar marker represents 1.5 μm. (b) Medulla oblongata at 7 days. Intermediate stages of virus multiplication in the cytoplasm of an oligodendrocyte can be seen. Bar marker represents 700 nm.
Table 2. *Multiplication of SFV strains in primary mouse brain cell cultures*  

<table>
<thead>
<tr>
<th>Virus</th>
<th>Glial cells</th>
<th>Neurons</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Virus yield† (p.f.u./10⁶ cells)</td>
<td>RNA synthesis‡ (c.p.m./10⁶ cells)</td>
</tr>
<tr>
<td>L10</td>
<td>2 × 10⁹</td>
<td>50690</td>
</tr>
<tr>
<td>M9</td>
<td>1 × 10⁹</td>
<td>66257</td>
</tr>
<tr>
<td>A7</td>
<td>4 × 10⁸</td>
<td>51782</td>
</tr>
<tr>
<td>Uninfected</td>
<td>4450</td>
<td></td>
</tr>
</tbody>
</table>

* Cells were infected at a multiplicity of 100 p.f.u./cell. The values given are the average of duplicate determinations in two separate experiments.
† Produced at 16 h post-infection.
‡ Cells were pulse-labelled with 10 μCi/ml [³H]uridine in 2 ml medium and in the presence of 5 μg/ml actinomycin D, from 2 to 10 h post-infection.

Infection when focal necrosis accompanied by polymorphonuclear leukocyte infiltration was present in the thalamus and hippocampus. No virus particles were seen at this stage.

For mice infected i.c. with A7, no lesions were seen in the three mice sampled at 5 days post-infection. Focal areas of spongy change with mononuclear leukocytes and cells with dense pyknotic nuclei were randomly distributed at 6 and 7 days post-infection in the white matter of the brain and spinal cord. Cells with pyknotic nuclei were also present in otherwise normal grey and white matter. The distribution of these lesions was similar to those present in M9-infected mice. Collapsed myelin sheaths and Wallerian degeneration were seen in the spinal cords of some mice. Intermediate stages in virus multiplication were seen in oligodendrocytes in areas of myelin vacuolation at 6 and 7 days post-infection (Fig. 1). Vacuoles and membranous profiles were common in the cytoplasm of these cells while some in addition contained myelin figures and numerous lysosome-like inclusions. The nuclei of some of these oligodendrocytes were pyknotic. Mice sampled at 14 days showed fewer pyknotic nuclei and less severe spongy change. Collapsed myelin sheaths were still present.

**Infection of mouse brain cell cultures**

Indirect fluorescent antibody staining of neuron and mixed glial cell cultures showed that neuron cultures were 100% pure, but mixed glial cultures contained 78% astrocytes, 13% oligodendrocytes, 1% neurons and 8% uncharacterized cells. Yields of virus and virus-specified RNA synthesis in cultures infected with the three strains of SFV are shown in Table 2. For neurons, viral RNA synthesis could only be detected for L10, and the yield of infectious virus was about ten times that of A7 or M9. No virus-specific protein synthesis could be detected in neuron cultures (gel not shown). For glial cells, viral RNA synthesis was greater than that obtained in neurons, and was equivalent for all three SFV strains. The only viral protein detected in glial cells was the core protein. This was easily detectable for L10 and M9, but barely detectable for A7. Other viral proteins were probably obscured by the background of host protein synthesis (Fig. 2). The yield of infectious virus produced by glial cells was greater than that produced by neurons for all three virus strains.

The cytopathic effect produced in these cultures by the three strains was complex. The microscopical appearance of a typical uninfected neuron culture is shown in Fig. 3(a). Following infection of neurons, the first observable effect for all three virus strains was the retraction of most cell processes and the formation of clumps of cells; following this many cells became bright in phase contrast (Fig. 3b, c). This occurred several hours earlier for L10 than for M9 or A7. For L10 disintegration of the culture occurred by 16 h post-infection, leaving only a few cells (Fig. 3d). For neurons infected with M9 or A7, slower disintegration of the culture occurred over several days. For glial cells, a typical uninfected culture is shown in Fig. 4(a). Following infection of glial cells, rapid disintegration of a proportion of cells occurred (Fig. 4b, c, d). This was followed by slow disintegration of the remainder of the cells over several days.
Fig. 2 Protein synthesis in cultured glial cells infected with L10, M9 and A7 SFV. Cells were pulse-labelled from 4 to 6 h after infection, with [*S]methionine then chased for 1 h. (a) L10; (b) mock-infected; (c) M9; (d) A7. The position of the virus core protein (C) is indicated.

Fig. 3. Effect of SFV infection in cultured neurons at 16 h post-infection. (a) Uninfected cells. (b to d) Infected cells: (b) A7; (c) M9; (d) L10. Bar marker represents 40 μm.
DISCUSSION

Our results for multiplication of the virulent L10 and less virulent A7 and M9 strains of SFV in the mouse may be compared with the experiments of Pusztai et al. (1971), who compared the multiplication of A7 and the virulent V13 strain. In both sets of experiments A7 produced a viraemia following i.p. injection which was of higher titre than that produced by the virulent strain. The magnitude and duration of the viraemia for A7 were similar in both sets of experiments. Also, the peak titres of virus in the brain and the times at which they were reached following i.p. injection were similar. For i.c. injection the titres of virus in the brain were ten- to 100-fold higher than for i.p. injection, although most mice survived in both sets of experiments. It is also apparent that both virulent strains behaved similarly in that multiplication to high titre occurred in the brain following both i.p. and i.c. injection resulting in the death of all infected mice. For the M9 strain, a viraemia was produced following i.p. injection which was similar to that produced by L10. The peak titre reached in the brain was higher for M9 than for A7 for both i.p. and i.c. injection, although lower than for L10. Other differences between M9 and A7 are that M9 produced clinical signs in a minority of mice following i.p. injection, and most mice infected i.c. with M9 died. For A7, most mice infected either i.p. or i.c. survive (this study and Bradish et al., 1971).
The results of these initial experiments indicated that, following i.c. injection, A7 multiplied in the brain to a titre higher than that of i.p. injected M9. Since virus can be detected in the brain by electron microscopy following i.p. injection of M9 (Atkins & Sheahan, 1982), but not after i.p. injection of A7, we examined mice injected i.c. with A7 in an attempt to determine the site of virus multiplication in the brain. Following i.c. injection, degenerating oligodendrocytes containing nucleocapsids and cytoplasmic vesicles could be detected for A7 at 6 days post-infection and neurons were not affected. One peculiarity following i.c. infection with A7 was that mature virions were rarely observed. The explanation for this is not clear at present. Bruce et al. (1984) have found that mixed glial cultures derived from rat brain become depleted of oligodendrocytes following A7 infection. Therefore, we conclude that for both M9 and A7, virus multiplication in the CNS results in damage to oligodendrocytes which triggers subsequent immune-mediated demyelination. The inability to detect the site of virus multiplication in the CNS of mice infected i.p. with A7 may be due to the insensitivity of electron microscopy.

We have shown that all three strains of SFV utilized in the present study multiply more efficiently in mixed glial cell cultures than in neurons. However, the glial cell cultures used in the present study were mostly astrocytes and contained only a minority of oligodendrocytes and other cells. Purification of oligodendrocytes will be necessary to study the interaction of SFV strains with these cells in culture. It is clear that the virulent L10 strain of SFV multiplies better in neurons than either the M9 or A7 strain. This supports our previous conclusion (Barrett et al., 1980) that the virulence of the L10 strain is determined by its greater ability to damage neurons.

We believe, therefore, that the evidence presented in this and our previous studies (Barrett et al., 1980; Atkins & Sheahan, 1982; Atkins, 1983; Sheahan et al., 1983; Gates et al., 1984), by Bruce et al. (1984) and by other workers (summarized by Atkins et al., 1985) suggests that acute demyelination produced by the M9 and A7 strains is immune-mediated, and is triggered by the infection of glial cells, probably oligodendrocytes. The demyelination involves the action of T-lymphocytes, since in nude mice it is less severe (Jagelman et al., 1978; Gates et al., 1984). For A7 and M9, damage to neurons is arrested by immune intervention before a lethal threshold of damage can be exceeded.

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SFV pathogenicity


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