The Pathogenicity of the M9 Mutant of Semliki Forest Virus in Immune-compromised Mice

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SUMMARY

The role of immune mechanisms in the pathogenicity of the M9 mutant of Semliki Forest virus (SFV) has been examined by the use of immune-deficient and immune-suppressed mice. In immune-competent BALB/c and C57BL/6 mice, the lesions in the central nervous system (CNS) were characterized by acute demyelinating meningoencephalomyelitis. Myelin vacuolation and demyelination were more severe in BALB/c mice than in mice with a C57BL background. The mortality was 12% and 29% respectively. Treatment with cyclophosphamide or sodium aurothiomalate did not greatly alter the type of lesion produced, although mortality was increased. Lesions were less severe in nude (T-lymphocyte-deficient) mice and more severe in beige (natural killer cell-deficient) mice than in most immune-competent mice. Mortality was marginally increased in nude mice but not in beige mice. Demyelination in nude mice was followed rapidly by remyelination. Immune modification did not significantly alter the titres of virus in the brain at 7 days post-infection and infectious virus had been cleared from the brain by 14 days in all cases. Degenerating oligodendrocytes were detected in the CNS of all immune-modified mice examined at day 7. This study therefore suggests that both immune mechanisms and destruction of oligodendrocytes play a role in the production of demyelination by M9.

INTRODUCTION

Demyelination of central nervous system (CNS) axons is a consequence of many virus diseases and is a feature of human diseases such as multiple sclerosis. Laboratory models of demyelination include virus infections (Dal Canto & Rabinowitz, 1982), chemical treatment (Blakemore, 1973) and experimental allergic encephalomyelitis (EAE), an autoimmune disease produced by injection of myelin components (Wisniewski et al., 1982). The study of such laboratory models has produced two theories to explain the mechanism of demyelination: one is that destruction of the myelin-producing cell, which in the CNS is the oligodendrocyte, leads to subsequent demyelination; the other is that demyelination is produced by an autoimmune response which involves the stripping of myelin by lymphocytes and macrophages. Some chemical treatments such as cuprizone (Blakemore, 1973) lead to selective destruction of oligodendrocytes and subsequent demyelination, whereas EAE is immune-mediated. The situation with virus infections is more complicated; the JHM strain of murine coronavirus, which produces a demyelinating disease in mice and rats, has been shown to exhibit a tropism for oligodendrocytes (Lampert et al., 1973; Nagashima et al., 1978). Multiplication of virus in oligodendrocytes has also been shown for a human demyelinating disease, progressive multifocal leukoencephalopathy (Mazlo et al., 1980). An autoimmune mechanism of demyelination has been suggested by Dal Canto & Rabinowitz (1982) from their studies of Theiler's murine encephalitis, vesicular stomatitis and Venezuelan equine encephalitis viruses.
Demyelination in mice produced by the alphavirus Semliki Forest virus (SFV) has been used as a model by ourselves and others. Most authors have postulated that demyelination by the avirulent A7 strain of SFV is immune-mediated (Jagelman et al., 1978; Suckling et al., 1978; Berger, 1980; Kelly et al., 1982). Using the neurovirulence mutants M9 and M136 of SFV (Barrett et al., 1980), we have demonstrated inflammatory demyelination with degenerative change and virus particles in oligodendrocytes (Sheahan et al., 1981, 1983; Atkins & Sheahan, 1982). Unlike in infection with the virulent wild-type L10 strain, neurons are not usually destroyed by these mutants and the majority of mice survive the infection. To investigate the role of the immune response in demyelination produced by M9, we have carried out studies with immune-suppressed and immune-deficient mice. The results indicate that demyelination is due to a cytolytic infection of oligodendrocytes and an associated immune response.

**METHODS**

**Virus.** The isolation and characterization of the M9 mutant of SFV, and the growth of working stocks of virus from single plaque isolates have been previously described (Barrett et al., 1980; Atkins & Sheahan, 1982). A single stock of virus, stored in aliquots at −70 °C, was used in all experiments. The dose in all animal inoculations was 3 × 10³ p.f.u. given intraperitoneally (i.p.) in 0.5 ml phosphate-buffered saline (PBS).

**Mice.** Inbred mice of BALB/c and C57BL genetic backgrounds were used. C57BL mice were of the C57BL/6 or C57BL/10 substrains. We have previously shown that C57BL/6 and BALB/c mice differ in their responses to M9 infection (Sheahan et al., 1983). All mouse strains were originally obtained from Olac (1976) Ltd. (Bicester, Oxfordshire, U.K.) and subsequently maintained as inbred colonies. Nude (nu/nu) mice were derived from crosses of heterozygous (nu/+)) females with homozygous (nu/nu) males. Breeding colonies and experimental nude mice were housed under barrier conditions in separate isolators. Beige (bg/bg) mice were obtained by crossing C57BL/6 (bg/bg) homozygotes. All experimental mice were 39 to 41 days old.

**Experimental design.** Initially, groups of 20 immune-modified or control mice were monitored daily for clinical signs and survival over a period of 14 days after infection with M9. Subsequently blood and brain samples from groups of six infected mice were titrated individually at 7 and 14 days after infection as previously described (Barrett et al., 1980). Brains and spinal cords from groups of three mice were removed at the same time intervals and examined by light and electron microscopy as described by Sheahan et al. (1981). These time intervals were chosen because in immune-competent BALB/c mice, virus is present in the brains of most mice at 7 days post-infection, but has been cleared from the blood. At day 14, virus is absent from both blood and brain but demyelination is most severe (Barrett et al., 1980; Atkins & Sheahan, 1982). In some experiments, pathological examinations were also carried out at days 5 and 21 post-infection and pathological examination of sodium aurothiomalate (SATM)-treated mice was carried out only at day 7, because of subsequent mortality. Statistical significance of numerical results was assessed at the 5% level, using standard error for time of death and the chi-squared test for percentage mortality.

**Immune suppression.** Cyclophosphamide (Sigma) was diluted in PBS and injected i.p. at a dose of 150 mg/kg body weight, 24 h before virus infection (Bradish et al., 1975a). SATM was obtained as ‘Myocrisin’ (May and Baker, Ltd., Dagenham, Essex, U.K.), and was injected i.p. at a dose of 350 mg/kg body weight, 24 h before virus infection (Bradish et al., 1975b).

**Neutralizing antibody.** This was titrated by mixing twofold dilutions of serum in PBS with 50 p.f.u. of virus and incubating at 37 °C for 30 min. The mixtures were then plaque-assayed. The titre of neutralizing antibody was taken to be the reciprocal of the serum dilution that gave a 50% reduction in plaque numbers when compared with a control incubated only with PBS. Tittrations were performed separately on groups of six mice sacrificed at 7 days post-infection.

**RESULTS**

**Clinical findings**

These are summarized in Table 1. For immune-competent mice infected with M9, most showed ruffling of the fur, lethargy and paresis beginning 5 days after infection. A proportion of all infected mice showed paralysis of one or more limbs; most immune-competent mice, including many which had earlier shown paralysis, appeared normal by 28 days after infection. Administration of cyclophosphamide or SATM greatly increased mortality in BALB/c and C57BL/6 mice infected with M9. Administration of cyclophosphamide or SATM alone did not
Table 1. Clinical findings*

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>Treatment</th>
<th>% Mortality</th>
<th>Mean time of death post-infection (days ± standard error)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c</td>
<td></td>
<td>12.0</td>
<td>10.3 (+ 1.1)</td>
</tr>
<tr>
<td>BALB/c</td>
<td>Cyclophosphamide</td>
<td>86.0</td>
<td>6.5 (+ 0.2)</td>
</tr>
<tr>
<td>BALB/c</td>
<td>SATM</td>
<td>90.0</td>
<td>6.0 (+ 0.6)</td>
</tr>
<tr>
<td>BALB/c (nu/nu)</td>
<td></td>
<td>31.5</td>
<td>9.7 (+ 0.9)</td>
</tr>
<tr>
<td>C57BL/6</td>
<td></td>
<td>28.8</td>
<td>8.9 (+ 0.5)</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>Cyclophosphamide</td>
<td>90.0</td>
<td>7.7 (+ 0.4)</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>SATM</td>
<td>87.5</td>
<td>7.8 (+ 0.5)</td>
</tr>
<tr>
<td>C57BL/6 (bg/bg)</td>
<td></td>
<td>36.2</td>
<td>8.8 (+ 0.4)</td>
</tr>
<tr>
<td>C57BL/10 (nu/+)</td>
<td></td>
<td>28.6</td>
<td>6.5 (+ 0.5)</td>
</tr>
<tr>
<td>C57BL/10 (nu/nu)</td>
<td></td>
<td>35.0</td>
<td>7.4 (+ 0.2)</td>
</tr>
</tbody>
</table>

* Forty-day-old mice were infected i.p. with 3 × 10^3 p.f.u. of the M9 mutant of SFV and observed for 14 days. Cyclophosphamide or SATM was given 24 h before infection.

M9 SFV infection

produce clinical signs. For the nude mutation, the effect on mortality was less severe and was statistically significant only for BALB/c (nu/nu) mice. The beige mutation had no effect on mortality for C57BL/6 mice.

Pathology

The lesions in the CNS of immune-competent BALB/c and C57BL/6 mice have been described previously (Sheahan et al., 1983).

Treatment with cyclophosphamide or SATM did not greatly alter the morphology or distribution of lesions in BALB/c or C57BL/6 mice compared to most immune-competent mice. In immunosuppressed BALB/c mice at 5 and 7 days, occasional hypertrophic and vacuolated oligodendrocytes in areas of myelin vacuolation contained virus nucleocapsids 25 to 30 nm in diam. (Fig. 1), but mature virus particles were uncommon. In contrast to untreated mice, stripping of myelin by macrophage processes was seen 7 days post-infection (Fig. 2). Oligodendrocytes containing virus were not seen in treated C57BL/6 mice. In SATM-treated mice of both strains, meningeal infiltrates included polymorphonuclear leukocytes and occasional small foci of neuronal necrosis were seen in the hippocampus and cerebral cortex. Lymphocytes, necrotic cells and occasionally macrophage-like cells with extensive profiles of smooth endoplasmic reticulum occurred in vacuoles in myelin sheaths (Fig. 3).

For BALB/c (nu/nu) mice focal spongiform lesions with occasional mononuclear leukocytes, necrotic cells and naked axons were randomly distributed mostly in white matter 5 and 7 days post-infection (Fig. 4, 6). These lesions were smaller and less severe than in most immune-competent mice examined at these times. Areas of myelin pallor and vacuolation with naked and remyelinating axons, were prominent at 14 days (Fig. 5, 7). As in immune-competent mice, lymphocytes and necrotic cells occurred in vacuoles in myelin sheaths. Small numbers of virus nucleocapsids were seen in vacuolated, hypertrophic oligodendrocytes in areas of myelin vacuolation at 5 days. For C57BL/10 (nu/nu) mice, focal areas of spongiform degeneration and necrosis, closely resembling but less severe than those in most immune-competent C57BL/6 or C57BL/10 (nu/+ ) mice, were present at 5, 7 and 14 days post-infection. Vacuolated oligodendrocytes were present in areas of myelin vacuolation but no virus was seen. As in immune-competent mice, myelin vacuolation and demyelination were less severe in C57BL/10 (nu/nu) mice than in BALB/c (nu/nu) mice.

For C57BL/6 (bg/bg) mice, necrosis, spongiform degeneration and leukocytic infiltration were consistently more severe at days 7 and 14 post-infection than in C57BL/6 mice. The lesions at 21 days were indistinguishable from those in C57BL/6 mice.

Virus titration

Virus was recovered from the brains of most immune-competent and immune-modified mice at 7 days post-infection but was absent from all mice at day 14. Immune modification had no
significant effect on the titres of virus recovered from the brain at day 7, which ranged in individual mice from $2.3 \times 10^4$ to $2 \times 10^7$ p.f.u./g. This high degree of variation was also found in our previous study of immune-competent BALB/c mice (Atkins & Sheahan, 1982). Virus was
M9 SFV infection

Fig. 4. BALB/c (nu/nu) mouse, spinal cord, 7 days post-infection. Myelin vacuolation, occasional mononuclear leukocytes and naked axons (arrows). Light micrograph; bar marker represents 4.0 μm.

Fig. 5. BALB/c (nu/nu) mouse, spinal cord, 14 days post-infection. Myelin vacuolation, dense pycnotic nuclei (arrows) and naked axons. Light micrograph; bar marker represents 6.7 μm.

Table 2. Serum neutralizing antibody response to M9 at 7 days post-infection

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>Treatment</th>
<th>Antibody titre*</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c</td>
<td></td>
<td>128-256</td>
</tr>
<tr>
<td>BALB/c</td>
<td>Cyclophosphamide</td>
<td>Undetectable</td>
</tr>
<tr>
<td>BALB/c</td>
<td>SATM</td>
<td>4-8</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>Cyclophosphamide</td>
<td>Undetectable</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>SATM</td>
<td>64-256</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>SATM</td>
<td>128-256</td>
</tr>
</tbody>
</table>

* Calculated as the reciprocal of the dilution giving 50% neutralization; the values given are the ranges from six individual mice whose sera were titrated separately.

absent from the blood of all groups of immune-competent, nude and beige mice at days 7 and 14. For cyclophosphamide treatment, a viraemia was present at day 7 for both BALB/c and C57BL/6 mice. This viraemia was significantly higher [7.6(±2.0) × 10^6 p.f.u./ml] for BALB/c mice than for C57BL/6 mice [1.8(±0.3) × 10^2 p.f.u./ml]. SATM treatment also induced a viraemia at day 7 in BALB/c mice [9(±4) × 10^3 p.f.u./ml] but no viraemia could be detected for C57BL/6 mice.

Neutralizing antibody responses

Since there were significant differences in viraemia at 7 days post-infection between BALB/c and C57BL/6 mice following treatment with SATM and cyclophosphamide, we measured neutralizing antibody titres in these two mouse strains following drug treatment (Table 2). It is clear that at this time there was no significant difference in antibody titres between BALB/c and C57BL/6 mice. Cyclophosphamide treatment inhibited antibody synthesis for both strains. However, SATM treatment reduced antibody titres for BALB/c but not for C57BL/6 mice.

DISCUSSION

We have examined the role of immune mechanisms in the pathogenicity of the neurovirulence mutant M9 of SFV, through the use of immune-suppressed and immune-deficient mice. The results indicate that several components of the immune system influence viral clearance, the severity of inflammatory demyelination, and mortality in this infection.

The inhibitory effect of cyclophosphamide on the function of B-lymphocytes and suppressor T-lymphocytes has been described (Turk & Parker, 1982). The immunosuppressive action of
cyclophosphamide in the present study was shown by reduced antibody titres and delayed viral clearance from the blood. Similar results were obtained in infected BALB/c mice treated with SATM, an inhibitor of macrophage function (Allner et al., 1974; Mehta & Webb, 1982) and
lymphocyte proliferation (Lipsky & Ziff, 1976). Mortality was greatly increased in BALB/c and C57BL/6 infected mice following either of these treatments but no significant effect was seen on brain virus titres or on the development of CNS lesions. The relatively mild lesions in most nude (T-lymphocyte-deficient) mice indicated that T-lymphocytes play an important role in the production of tissue damage in this infection. Lesions in nude mice contained fewer macrophages and remyelination occurred faster than in the immune-competent animals. Furthermore, the increased severity of early lesions in beige (natural killer cell-deficient; Roder & Dowe, 1979) mice indicated that natural killer cells play a defensive role against M9 infection but have little effect on the demyelination process.

The most outstanding feature of the study, however, was the finding that M9 exerts a direct cytotoxic effect on oligodendrocytes not only in immune-competent mice but also in immune suppressed and immune-deficient mice. This finding contrasts with the many studies by other workers using the avirulent A7 strain of SFV, where the demyelination appears to be immunologically mediated and where direct viral cytolysis has not been found (Jagelman et al., 1978; Suckling et al., 1978; Berger, 1980; Fazakerley et al., 1983). Other findings reported here in immune-suppressed and nude mice infected with M9 also contrast with those reported in similar studies of mice infected with A7 although the times of administration of immune-suppressive drugs varied. Treatment of A7-infected mice with cyclophosphamide has been shown to delay the development of CNS lesions and clearance of virus from the brain, to elevate brain virus titres and to result in a significant reduction in serum haemagglutination inhibition antibody titres (Suckling et al., 1977; Berger, 1980). In contrast to untreated mice, SATM treatment allowed fully coated virus to be seen in the brain lesions (Mehta & Webb, 1982). While most workers agree that nude mice infected with A7 show only mild microcystic lesions with no demyelination (Jagelman et al., 1978; Fazakerley et al., 1983) or mild spongiform degeneration (Berger, 1980), Chew-Lim (1979) found that the T cell deficiency resulted in more widespread demyelination than in immune-competent mice. Nude mice infected with A7 also showed delayed viral clearance from the brain resulting in a persistent subclinical CNS virus infection (Jagelman et al., 1978). A7 and M9 have been shown to have similar molecular and host range properties (Atkins, 1983) but further studies are obviously required to explain the differences in pathogenicity between these two viruses.

The findings discussed here confirm previous reports that genetic differences between different strains of mice influence the pathogenesis of SFV infection (Suckling et al., 1980; Sheahan et al., 1983). We have shown that myelin vacuolation and demyelination were more prominent in BALB/c mice than in C57BL/6 mice. Similarly, virus particles were commonly observed in degenerating oligodendrocytes in mice of a BALB/c background, were seen only rarely in immune-competent C57BL/6 mice and were not found in C57BL/10 (nu/nu) mice. It is also apparent that BALB/c mice are more susceptible to immunosuppression, since treatment with SATM inhibited viral clearance and antibody synthesis in BALB/c but not in C57BL/6 mice. Other workers have found that BALB/c, but not C57BL/6 mice are susceptible to EAE following low doses of cyclophosphamide (Lando et al., 1980), and that BALB/c mice have a lower phagocytic index than C57BL/6 mice (Otu et al., 1976). This may mean that BALB/c mice have a less efficient immune response than C57BL/6 mice, but the reason for this is not clear at present.

This study shows that oligodendrocyte damage and demyelination occur in immune-suppressed and immune-deficient mice infected with M9, and this is consistent with our hypothesis (Sheahan et al., 1983) that the trigger for demyelination in this system is the cytolytic infection of oligodendrocytes. It is possible that the release of myelin antigens from damaged oligodendrocytes triggers an immune response involving T lymphocytes and macrophages. Further studies are in progress to assess the significance of the lymphocyte and macrophage responses in this system including the effect of these cells on the remyelination process.

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