In Semliki Forest virus encephalitis, antibody rapidly clears infectious virus and is required to eliminate viral material from the brain, but is not required to generate lesions of demyelination

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Semliki Forest virus (SFV) infection of the laboratory mouse provides a well-characterized tractable system to study the pathogenesis of virus encephalitis and virus induced demyelination. In μMT mice, which have no antibodies, infectious virus persisted in both the serum and the brain for several weeks, indicating that antibodies are required to eliminate infectious virus. In immunocompetent mice, virus infectivity in the brain was undetectable after the first week of infection, but virus RNA levels declined slowly. Following SFV infection, lesions of demyelination were present in the brains of both immunocompetent and μMT mice, indicating that antibodies are not required to generate lesions of demyelination.

Studies using in situ hybridization and PCR have detected alphavirus RNA in the brains of immunocompetent mice many weeks after infection (Donnelly et al., 1997; Levine & Griffin, 1992). Infectivity assays only determine levels of infectious virus above a limit of detection and only detect infectious virus in excess of the neutralizing capacity of the homogenized tissue sample. Absence of infectious virus, as detected in an infectivity assay, therefore does not necessarily equate to elimination of all infectious virus or of virus material capable of giving rise to infectious virus. In this study, virus clearance was determined both by infectivity assay and by quantitative RT-PCR (q-PCR) to assess virus RNA load.

Groups (n=12) of 129×C57BL/6-μMT (μMT) and 129×C57BL/6 (wt) mice were inoculated intraperitoneally with 5000 p.f.u. of the avirulent A7(74) strain of SFV. All mice were bred and maintained in the Centre for Infectious Diseases Animal Unit, College of Medicine & Veterinary Medicine, University of Edinburgh, UK. Animals were kept in HEPA-filtered boxes in specific pathogen-free and
environmentally enriched conditions with a 12 h light–dark cycle and food and water supplied ad libitum. All breeding and experimental studies were agreed by the University of Edinburgh Ethical review Committee and were carried out under the authority of a UK Home Office license. All animals were used between 4 and 5 weeks of age. Three mice were sampled at 4 days and 2, 4 and 8 weeks post-infection. Serum samples and one half of each brain were used to titre infectivity. The other half of each brain was divided into two and used to extract RNA and to study neuropathological changes.

At 4 days and 2, 4 and 8 weeks post-infection, infectious virus was detectable by standard plaque assay (Fazakerley et al., 1993) in the sera of all μMT mice, indicating a persistent plasma viraemia (Fig. 1a). The serum virus infectivity titres ranged from 2.6 to 4.8 log_{10} p.f.u. ml^{-1}. The range was similar at 2, 4 and 8 weeks. In contrast, by 4 days and all times thereafter all wt mice had infectivity titres below the limit of detection (Fig. 1a); this is consistent with previous studies (Fazakerley et al., 1993). In the brain, high levels of infectious virus were detectable at 4 days in both μMT and wt mice (Fig. 1b). In μMT mice, infectious virus remained detectable for 8 weeks, albeit at considerably reduced levels; all mice sampled between 2 and 8 weeks post-infection had detectable levels of infectious virus (Fig. 1b). Titres ranged from 2.4 to 5.4 log_{10} p.f.u. g^{-1} brain. In contrast, from 2 weeks post-infection, infectious virus in the brains of all wt mice were below the limit of detection (Fig. 1b). We conclude that antibodies are required to clear infectious virus from both the blood and the brain.

To determine whether clearance of infectious virus correlated with the elimination of virus RNA, at 2, 4 and 8 weeks post-infection, levels of virus RNA in the brain were measured by q-PCR. Brain tissue samples were submerged in RNA later. RNA was extracted using a Qiagen RNeasy Lipid mini-kit according to the manufacturer’s instructions. cDNA was produced from total RNA using Superscript II RNase H^- Reverse Transcriptase and amplified with primers targeting a 173 bp fragment of the SFV E1 structural gene. q-PCR was performed using FastStart DNA Master SYBR Green I kit (Roche). An initial denaturation step at 95 °C for 10 min was followed by 40 cycles of amplification. Each cycle comprised denaturation at 94 °C for 10 s, annealing at 62 °C for 5 s and extension at 72 °C for 10 s. The Tm of the 173 bp product was approximately 88.5 °C. As a standard positive transcript for PCR analysis, an in vitro transcript from the pGEM1-SFV cDNA plasmid containing the structural genes of SFV was transcribed using a Promega Ribomax kit. For quantification, serial dilutions of this plasmid were assayed to produce a standard curve. Virus RNA was detectable in all μMT and 87% of wt mice (Fig. 1c). At each time point, μMT mice had higher levels of viral RNA than wt mice. The difference between the two mouse strains was 20-fold at 2 weeks and 50-fold at 8 weeks. Virus loads were compared using the Mann–Whitney test and were

Fig. 1. Infectious virus measured by plaque assay in SFV-infected μMT (●) and 129×C57BL/6 (○) mice in serum (a) and brain (b). Titres were measured by plaque assay in BHK-21 cells. SFV virus RNA titres in brain tissue were measured by q-PCR (c). Each point represents the mean of two replicates. The horizontal bars for each group represent the mean value for all samples. The limit of detection is indicated by the dashed line. In (c) at each time point there was a significant difference between the two animal groups using Mann–Whitney test (P<0.05). Comparison of brain virus titres and RNA levels in individual mice demonstrated no correlation; for the μMT mice r^2=0.16 and for the 129×C57BL/6 mice r^2=0.26.
significantly different ($P<0.05$) at all time points. In μMT mice there was no correlation between infectious virus titres (serum or brain) and levels of brain virus RNA. Levels of virus RNA in the brain declined with time in both mouse strains. At 8 weeks post-infection, >6 weeks after infectious virus was no-longer detectable, 3/4 wt mice still had virus RNA detectable in the brain.

Previous studies have shown that between 2 and 4 weeks post-infection SFV A7(74)-infected mice have lesions of inflammatory demyelination in the brain and that these lesions require adaptive immune responses, including CD8 T cells (Subak-Sharpe et al., 1993). To determine whether the lesions of demyelination also require antibodies, the brains of the three μMT and three wt mice were sampled at 2 weeks post-infection. Brains were fixed by immersion in 10% phosphate buffered formal saline for at least 48 h, processed through paraffin wax, stained with luxol fast blue (LFB) and cresyl fast violet (Kluver & Barrera, 1953) and coded prior to examination. At least three sections from three separate areas of each brain were studied and scored for the extent of inflammation and demyelination (Table 1). The brains of mice from both mouse strains showed microcystic changes, mononuclear cell inflammatory infiltrates, which were mostly perivascular, and lesions of demyelination (Fig. 2). We conclude that antibodies are not required to generate lesions of demyelination.

### Table 1. The extent of inflammation and demyelination in the brains of the μMT and 129×C57BL/6 mice sampled at 2 weeks post-infection

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>Demyelination</th>
<th>Inflammation</th>
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<tbody>
<tr>
<td>129×C57BL/6</td>
<td>0.8 (0–3)</td>
<td>1.4 (0–3)</td>
</tr>
<tr>
<td>μMT</td>
<td>1 (0–3)</td>
<td>2 (1–4)</td>
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</tbody>
</table>

At least nine coded sections representing three different areas of each brain were studied microscopically, scored and a mean score obtained. The values given are the mean scores from three mice. The values in parentheses show the range of the scores for individual sections. Inflammation and demyelination were scored as follows: 0, no inflammation; 1, one or few foci of inflammation with a few mononuclear cells apparent, either around blood vessels or invading the parenchyma; 2, several such small foci, or one or two foci containing several mononuclear cells; 3, one or a few large foci of inflammation with many mononuclear cells and 4, many areas of inflammation throughout the brain including at least one area with large inflammatory infiltrates. For demyelination the respective scores were: 0, no demyelination; 1, one or more small foci of microcystic change within the white matter; 2, one or more small foci of microcystic change within the white matter with clear loss of LFB staining; 3, one or more medium-sized foci showing clear loss of LFB staining and 4, one or more large foci showing clear loss of LFB staining.

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**Fig. 2.** Black and white image of luxol fast blue stained 5 μm paraffin sections counterstained with cresyl fast violet. (a–d) SFV-infected μMT mouse brain sections 2 weeks post-infection. (a) Blood vessel (bv) surrounded by infiltrating mononuclear cells, an example of this is indicated by the arrow. Elongated cells surrounding the bv show characteristic morphology of macrophages. (b) Microcystic change seen as tissue vacuolation in the μMT mouse brain following SFV infection. Mononuclear cell infiltrates are indicated by arrows. (c) White matter tract with an area of demyelination enclosed by the circle adjacent to an area with normal myelination (n) as shown by darker (luxol fast blue) staining. Arrow indicates inflammatory infiltrates. (d) An area of demyelination (enclosed within circle) adjacent to unaffected white matter tract (n). A colour version of this figure is available in JGV Online.
In immunocompetent mice, SFV A7(74) infectivity in the blood is only detectable in the first 3 days and in the brain in the first 10 days (Fazakerley et al., 1993). The present study shows, for the first time, the temporal course of virus RNA levels in the brain. In the brains of immunocompetent (C57BL/6) mice, as expected, infectious virus was cleared within 2 weeks; however, levels of virus RNA declined slowly. The levels of virus RNA were not quantified; however, the presence of alphavirus RNA months after infection in the brains of immunocompetent mice has also been reported for the M9 strain of SFV and the AR339 strain of Sindbis virus (Donnelly et al., 1997; Levine & Griffin, 1992).

In the brains of SFV-infected μMT mice, following an early reduction in levels of infectious virus, infectious virus and virus RNA remained detectable throughout the 12 weeks of the study. There was considerable variation in titres between mice. Persistence of infectious virus for months in the brain is also observed in athymic nu/nu and SCID mice infected with SFV A7(74) and, as in the current study, levels of infectious virus show considerable variation between individual mice (Amor et al., 1996; Fazakerley et al., 1993). The TE strain of Sindbis virus has also been observed to persist for weeks in the brains of SCID and μMT mice (Burdeinick-Kerr et al., 2007; Levine et al., 1991). Clearly, at least for alphaviruses, antibody is required to clear infectious virus from the CNS. The explanation for the high variability in SFV infectivity titres between mice and for the variability between levels of infectious virus and virus RNA is not clear. One factor may be persistence of virus RNA that cannot give rise to infectious virus; another may be that levels of infectious virus, but not virus RNA, rise and fall in response to some defence system, perhaps interferons.

Both immunocompetent and μMT mice sampled at 2 weeks post-infection had CNS inflammation and demyelination. The extent of both was less than that observed in our previous studies with the same strain of SFV in BALB/c mice (Fazakerley et al., 1983; Subak-Sharpe et al., 1993). This is most likely to reflect genetic differences between the mouse strains, which for C57BL/6 and BALB/c have been demonstrated to affect immune responses in many systems and which can affect the course of SFV encephalitis (Suckling et al., 1980). Interestingly, inflammation was more extensive in the μMT mice than in wt mice. Most likely, this is because in the absence of antibodies more virus-infected cells resulted in more antigen and more chemokines driving the inflammation; demyelination also had a slightly higher score consistent with more infected oligodendrocyte targets of CD8+ T cells. Our previous studies showed that demyelination requires CD8+ T cells; however, these studies did not establish whether antibodies were also required (Subak-Sharpe et al., 1993). Studies by others (Mokhtarian et al., 2003) suggest that B cells and anti-myelin antibodies also contribute to myelin pathology. The current study makes it clear that T-cell responses are sufficient and that antibodies are not required.

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References


