Role of immune responses in protection and pathogenesis during Semliki Forest virus encephalitis

Sandra Amor, Martina F. Scallan, Margaret M. Morris, Heather Dyson and John K. Fazakerley

The course of Semliki Forest virus (SFV) A7(74) infection in immunocompetent BALB/c, athymic nu/nu and severe combined immunodeficient (SCID) mice was compared. BALB/c mice remained healthy and exhibited transient viraemia and infectious virus in the brain from days 2 to 7. Antibodies were detectable by day 5. In comparison, SCID mice displayed a high incidence of paralysis and died: the average day of death was day 23. From infection until death, virus was present in blood and brain. No antibodies were detectable. Athymic mice were intermediate with a transient viraemia and a persistent (> 210 days) sub-clinical central nervous system (CNS) infection. These mice produced anti-viral IgM but not IgG. The pattern of infection in BALB/c or nu/nu mice could be recreated in infected SCID mice by transfer of immune serum from BALB/c or nu/nu mice, with the important exception that although BALB/c immune serum could abolish infectivity titres in the CNS, scattered cells positive for viral RNA remained. Transfer of serum decreased mortality and delayed the onset of paralysis. Transfer to infected SCID mice of a non-neutralizing IgG anti-E2 monoclonal antibody did not affect the viraemia but could also reduce brain virus titres. Irrespective of specific immune responses, virus replication in CNS cells was restricted, was generally non-cytopathic and in the absence of specific immune responses could persist. From day 14 lesions of inflammatory, primary demyelination were observed throughout the CNS of BALB/c mice. In contrast, despite prolonged brain virus titres, no demyelinating lesions were observed in infected nu/nu or SCID mice. Lesions could be initiated in the latter by transfer of spleen cells but not antibody. In summary, the focal restricted infection in the CNS of adult mice infected with SFV A7(74) is independent of specific immune responses. IgM antibodies clear the viraemia. IgG antibodies including non-neutralizing antibodies reduce and clear infectious virus but cells positive for viral RNA remain. These may normally be cleared by T cell responses which are damaging and give rise to lesions of demyelination.

Introduction

Experimental infection of laboratory animals with Semliki Forest virus (SFV), a member of the family Togaviridae, is widely used as a model to study mechanisms of virus-induced central nervous system (CNS) disease. After intraperitoneal (i.p.) inoculation of immunocompetent BALB/c or Swiss Aγ2G mice with the avirulent A7(74) strain of SFV, the virus replicates in muscle tissue giving rise to a transient plasma viraemia (Pusztai et al., 1971). Virus crosses cerebral endothelial cells to initiate small perivascular foci of infection in CNS cells, including neurons and oligodendrocytes (Pathak & Webb, 1980; Fazakerley et al., 1993). Infectious virus is not detectable after 10 days by which time focal areas of cellular infiltration are well established and primary demyelination is first observed. These lesions of demyelination are scattered throughout the CNS (Suckling et al., 1978; Kelly et al., 1982) and are maximal between 14 and 21 days. In contrast, lesions of demyelination do not develop in infected athymic nu/nu mice, despite life-long persistence of virus in the brain (Jagelman et al., 1978; Fazakerley et al., 1983; Fazakerley & Webb, 1987b). Reconstitution of nu/nu mice with splenocytes from nu/+ littermates restores the lesions of demyelination indicating that these are immune mediated (Fazakerley et al., 1983). Depletion of T cells but not B cells from the transferred population abrogates their ability to initiate demyelination (Fazakerley & Webb,
1987b). In vivo depletion of CD8+ cells prevents demyelination (Subak-Sharpe et al., 1993). Persistence of virus in brains of mu/nu mice without apparent damage to CNS cells may be a consequence of restricted virus replication in mature CNS cells (Fazakerley et al., 1993).

To study the pathogenesis of this infection in the complete absence of specific immune responses, mice with severe combined immunodeficiency (SCID) were infected with SFV and the course of infection compared to that in athymic mu/nu mice, which lack T cells, and immunocompetent BALB/c mice. SCID mice have an autosomal recessive defect that impairs VDJ rearrangement of immunoglobulin and T cell receptor genes (Schuler et al., 1986; Kim et al., 1988; Malynn et al., 1988). They display severe lymphocytopenia and have no detectable serum immunoglobulins or functional T and B cells, although myeloid cell differentiation and function are normal. Macrophages and NK cells are phenotypically and functionally intact and animals are able to synthesize interferon-γ and tumour necrosis factor (Bancroft et al., 1987, 1989). SCID mice have been used to study immune responses in bacterial (Wherry et al., 1991) and viral infections (Chow et al., 1992; Rozengurt & Sanchez, 1993) including that of the closely related Sindbis virus (Levine et al., 1991; Levine & Griffin, 1992).

Our study shows that in the absence of specific immune responses SCID mice infected with SFV A7(74) have a persistent viraemia, a persistent and restricted CNS infection and no lesions of demyelination. Comparison of the infection to that in mu/nu and BALB/c mice and studies on the transfer of immune sera indicate that IgM antibodies clear the viraemia but not the brain virus and that infectious brain virus can be reduced by IgG antibodies including a non-neutralizing anti-E2 IgG monoclonal. IgG antibodies can abolish infectivity titres in the brain but cannot remove all viral RNA. Lesions of demyelination are T cell mediated and CNS infection is restricted to small foci irrespective of specific immune responses.

**Methods**

*Mice.* CB17 [H-2b] scid/scid (SCID) mice were obtained from Bantin and Kingman, UK, and maintained in the Rayne Institute, St Thomas' Hospital, under specific-pathogen-free conditions. As we were unable to obtain immunocompetent CB17 mice, BALB/c mice (Harlan Olac, UK), which also have the H-2b haplotype, were used as controls. Mice of either sex were used at 5–6 weeks of age. Athymic BALB/c mu/nu mice (Harlan Olac, UK) were maintained in the Rayne Institute. St Thomas’ Hospital, under specific-pathogen-free conditions or housed in the Department of Pathology, University of Cambridge.

*Virus inoculation.* The A7(74) strain of SFV was originally obtained from C. J. Bradish (Microbiological Research Establishment, Porton Down, Salisbury, Wiltshire, UK) and was passed once through mouse brain and stored at −70 °C in aliquots as a 10−2 (w/v) dilution in PBS with 0.75% BSA (PBSA). Mice were inoculated i.p. with 5000 p.f.u. virus in 0.1 ml PBS. After inoculation mice were checked daily for clinical signs.

**Experimental protocol.** Forty BALB/c, 40 SCID and 26 mu/nu mice were infected and two to five mice from each group sampled at various time points between 2 and 32 days post-infection. Blood was diluted 10% in PBS. Brains were removed and bisected sagittally. Spinal cords were extracted under hydrostatic pressure by severing the lower lumbar column and inserting a 21 gauge needle attached to a syringe containing sterile PBS. Diluted blood, serum and half of each brain were stored at −70 °C prior to titration for virus or antibody. Virus titration was by plaque assay on monolayers of subconfluent BHK cells, as described previously (Fazakerley et al., 1993). The other half of each brain, pieces of spinal cord and other tissues were either frozen, cut on a cryostat and used for immunostaining or fixed in 5% formol saline, processed for paraffin histology and 5-µm sections either stained with haematoxylin and eosin or luxol fast blue for pathological assessment or processed to determine viral RNA by in situ hybridization.

In a separate series of experiments, SCID mice received sera pooled from BALB/c or mu/nu mice. This pooled sera was taken 7 days after a single inoculation of SFV or at day 35 after SFV inoculation at days 0, 14 and 28 (immune serum). Further groups of SCID mice received a monoclonal IgG2a, anti-SFV E2 (AKS-307) antibody (Khalili-Shirazi et al., 1986) which was concentrated from tissue culture supernatant by protein-G affinity chromatography. In a pilot study the half-life of BALB/c immune serum administered to SCID mice was found to be 3 days as measured by ELISA. Thus, in order to maintain antibody levels in recipient mice 0.1 ml serum was administered on days 2, 4 and 7 after SFV inoculation. Another group of SCID mice received single cell suspensions containing 4 × 10⁷ spleen cells taken 7 days after SFV infection of BALB/c mice.

*Antibody titres.* Total immunoglobulin was measured in serum by ELISA as described previously (Fazakerley et al., 1993). Values were considered positive when they exceeded the mean plus 3 st of sera from five uninfected BALB/c mice.

Neutralization titres were determined as the reciprocal of the dilution of serum capable of neutralizing 50 p.f.u. of virus when 0.2 ml of serum dilution was incubated with 0.2 ml PBS containing 100 p.f.u. SFV A7(74) and incubated for 1 h at room temperature. Plaques were determined by titration in duplicate on BHK cell monolayers as described previously (Fazakerley et al., 1993).

**In situ hybridization.** In situ hybridization was done on paraffin sections using an SFV specific probe labelled with 35S or digoxigenin as described previously (Fazakerley et al., 1993). Sections were counterstained with dilute haematoxylin and eosin or neutral red. No signal was obtained using this probe on similarly prepared sections of Thiel’s murine encephalomyelitis virus (TMEV) infected mouse brain or using a probe to TMEV on sections of SFV infected brains (Simas et al., 1995). Distribution of virus in the CNS was determined from autoradiographic images developed on Hyperfilm β-max (Amersham). After processing through photographic emulsion the same sections were observed microscopically. RNA positive cells were visualized in sections hybridized with a digoxigenin labelled probe using a sheep anti-digoxigenin antibody; a rabbit anti-sheep antibody conjugated to biotin, avidin-biotin-horseradish peroxidase amplification (ABC kit, Vector Laboratories) and diaminobenzidine as substrate. Sections were counterstained with haematoxylin and eosin.

*Immunocytochemistry.* All antibodies were diluted in PBS containing 5% normal mouse serum (NMS). The primary antibodies were rat monoclonal anti-F4/80 (clone CI.A3-1, Serotec), a phenotypic marker for monocytes, macrophages and microglial cells or anti-Lyt1 (clone
Immune responses in SFV encephalitis

YTS 121.1, Seralab) which detects CD5 on T lymphocytes. Cryostat sections (5–10 μm) were prepared from brains and cords of infected and non-infected SCID and BALB/c mice and mounted on slides previously treated with a solution of 0.05% gelatin, 0.25% chromic potassium sulphate (alum) and 0.03% sodium azide. Endogenous peroxidase activity was blocked using 0.03% H2O2 in PBS. Sections were placed in a humid chamber at room temperature and incubated sequentially with primary antibody (1 h), biotinylated rabbit anti-rat Ig, avidin–biotin–peroxidase complex (30 min) and diaminobenzidine chromogen (Sigma). Between each reagent sections were washed three times in PBS. Finally, sections were stained with haematoxylin. Controls in which each of the reagents was replaced with PBS were consistently negative.

Results

Course of infection

All infected BALB/c and nu/nu mice remained healthy throughout the course of the study. At various times after post-inoculation day 5, SCID mice became inactive, lost weight and developed ruffled fur, hunched posture and hind-limb paralysis. Nine out of the 40 SCID mice infected died before sampling, the first on day 5 and the others sporadically throughout the experiment. In a study of eight infected SCID mice (from which none were sampled) the average day of death (Semenov et al., 1975) was 22.7 days.

In BALB/c mice, viraemia was detectable from days 2 to 4 and virus was detectable in the brain by plaque assay from days 2 to 7 (Fig. 1a). No virus was detectable by infectivity assay in any of 18 mice studied between days 14 and 32. In nu/nu mice, viraemia was detectable from days 2 to 4 and brain virus from days 2 to 210 (Fig. 1b). In contrast, in SCID mice virus was detectable in both blood and brains throughout the course of the study (<32 days). Viraemia titres were as high as 10^4.7 p.f.u./ml on day 14 and titres of virus in the brain reached 10^5.7 p.f.u./g (Fig. 1c).

Titres of total anti-SFV immunoglobulin were determined by ELISA (Fig. 2). Antibody was first detectable at day 5 in infected BALB/c mice and increased rapidly until day 9 after which titres remained high until at least day 32. Anti-viral IgM was detectable in nu/nu mice between days 4 and 14 with a peak titre at day 6. No anti-viral IgG was detectable. As expected, no specific antibody, either IgM or IgG, was detectable in infected SCID mice.

Adoptive transfer of spleen cells, immune serum and monoclonal antibodies

The transient viraemia and persistent CNS infection in nu/nu mice (Fig. 1b), which produced only anti-viral IgM (Fig. 2b), suggested that IgM antibodies were responsible for clearance of the viraemia but, at least in the absence of an inflammatory response, could not clear the CNS infection. To determine the role of antibodies in virus clearance, groups of SFV infected SCID mice were inoculated with sera pooled from nu/nu or BALB/c mice at day 7 (d7) post-infection, BALB/c immune serum, an anti-E2 (AKS-307) monoclonal antibody or, as a control, pooled sera from BALB/c mice 7 days after infection with the BeAn strain of TMEV (Simas et al., 1995). All mice received 0.1 ml of serum or monoclonal antibody i.p. at days 2, 4 and 7 post-infection. The
antibody titres of these preparations by ELISA and neutralization assay are shown in Table 1. The monoclonal antibody had an ELISA titre almost as high as the pooled d7 BALB/c serum, but was non-neutralizing. Three mice from each group were sampled at days 4, 7 and 34 after infection and blood and brain virus titres determined (Table 2).

Transfer of BALB/c immune serum to infected SCID mice rapidly and completely cleared the viraemia. By plaque assay, no infectious virus could be detected in the CNS at days 7 or 34. All mice remained healthy and survived the infection. Likewise, after transfer of d7 serum (containing both IgM and IgG) from BALB/c mice the viraemia was cleared by day 4. This was maintained at day 34 and the mice showed no signs of paralysis. At days 4 and 7 there was a reduction in the infectious brain virus titre although this was transient since by day 34, 27 days after the final inoculation of serum, high brain virus titres were again present. As with d7 BALB/c serum, transfer of d7 serum from nu/nu mice (containing IgM but not IgG) also cleared the viraemia by day 4. However, this serum had no effect at all on the brain virus titre and in contrast to d7 or BALB/c

Table 1. ELISA and neutralization titres of antibodies

<table>
<thead>
<tr>
<th>Sera</th>
<th>IgM</th>
<th>IgG</th>
<th>Neutralization titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c immune serum</td>
<td>160</td>
<td>10240</td>
<td>2560</td>
</tr>
<tr>
<td>BALB/c d7 serum</td>
<td>320</td>
<td>2560</td>
<td>1280</td>
</tr>
<tr>
<td>nu/nu d7 serum</td>
<td>320</td>
<td>40</td>
<td>320</td>
</tr>
<tr>
<td>AKS-307</td>
<td>&lt; 10</td>
<td>1280</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>TMEV d7 serum</td>
<td>&lt; 10</td>
<td>&lt; 10</td>
<td>&lt; 10</td>
</tr>
</tbody>
</table>

Table 2. Virus titres in the blood and brain of SFV infected SCID mice after adoptive transfer of antibodies or spleen cells

<table>
<thead>
<tr>
<th>Virus titre</th>
<th>Blood</th>
<th>Brain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 4</td>
<td>Day 7</td>
</tr>
<tr>
<td>No serum</td>
<td>5.0</td>
<td>3.9</td>
</tr>
<tr>
<td>BALB/c immune serum</td>
<td>&lt; 1.7</td>
<td>&lt; 1.7</td>
</tr>
<tr>
<td>BALB/c d7 serum</td>
<td>1.8</td>
<td>&lt; 1.7</td>
</tr>
<tr>
<td>nu/nu d7 serum</td>
<td>19.1</td>
<td>16.4</td>
</tr>
<tr>
<td>AKS-307</td>
<td>4.2</td>
<td>3.2</td>
</tr>
<tr>
<td>SFV d7 spleen cells</td>
<td>2.5</td>
<td>&lt; 1.7</td>
</tr>
<tr>
<td>TMEV d7 serum</td>
<td>4.8</td>
<td>4.0</td>
</tr>
</tbody>
</table>
immune serum could not maintain clearance of the viraemia which was detectable again on day 34. By day 44, two mice had died and one was paralysed. In a second experiment SCID mice were given mu/nu sera (0.1 ml i.p.) over a longer time course, every second day from 2 to 14 days post-infection. Clearance of the viraemia was maintained for longer (> day 34). However, despite this prolonged antibody treatment the mu/nu serum still had no effect on brain virus titres. As with d7 BALB/c serum, the monoclonal anti-E2 transiently reduced the brain virus titre but, in contrast to both the d7 BALB/c and nu/nu sera, this non-neutralizing monoclonal IgG2a antibody had no effect on the viraemia. As expected, the anti-TMEV serum did not affect either blood or brain virus titres. Transfer of spleen cells taken from donor BALB/c mice at day 7 post-infection cleared infectivity titres in both blood and brain although more slowly than did antibody transfers. By day 34, absence of infectivity titre was maintained in both blood and brain.

Distribution of viral RNA determined by in situ hybridization

Random sections of brain (sagittal), spinal cord (transverse), heart, lung, liver, kidney, gut, spleen, lymph nodes, pancreas, thyroid and skeletal muscle were examined for the presence of viral RNA by in situ hybridization. In BALB/c mice, viral RNA was detected in brains and spinal cords of all mice sampled from days 2 to 11 and had a scattered, focal distribution (Fig. 3) as described previously (Fazakerley et al., 1993). Neither the size nor the number of foci increased after day 4. This coincided with termination of the viraemia. With the exception of a few positive skeletal muscle fibres at day 2, no positive signal was observed in any of the extraneural tissues of BALB/c mice examined at days 2 and 4 post-infection. The same pattern of infection was observed in mu/nu mice (Fig. 3), except that scattered foci of virus positive cells in the CNS remained detectable throughout the course of the study (day 210). In SCID mice, viral RNA was detected in skeletal muscle, myocardium, brain and spinal cord from days 4 to 32. Positive cells were not observed in any other tissues. As in BALB/c and mu/nu mice the CNS infection in SCID mice consisted of scattered foci; however, in contrast to BALB/c mice the number but not the size of these foci increased with time (Fig. 3). Microscopically, it was apparent that many of the foci in BALB/c, mu/nu and SCID brains were centred around blood vessels (Fig. 4A). Foci of RNA positive cells could be detected in mu/nu mice even as late as 210 days post-infection.

Study of the brains of the infected SCID mice given transfers of antibody or cells demonstrated a similar picture to the virus plaque assays: in general, where virus was detectable by plaque assay (Table 2) it was also apparent by in situ hybridization. There was, however, one important difference: small foci of viral RNA positive cells were consistently detectable at days 7 and 34 in the brains of all six SCID mice given BALB/c immune serum. No virus was detectable by plaque assay in any of these mice at this time.

Pathology

Foci of mononuclear cells were apparent in the brain and spinal cord of infected BALB/c mice from days 5 to 28. At times when viral RNA positive cells could be detected by in situ hybridization, between days 5 and 11, these were often observed within the inflammatory foci (Fig. 4B). Between days 5 and 7, at the times of highest CNS virus titre, virus positive cells without a surrounding inflammatory response and inflammation without virus positive cells were rare. The foci of inflammation were usually perivascular reflecting both the virus distribution and the source of the inflammatory cells. Immunolabelling indicated that the inflammatory response contained both Lyt1+ T lymphocytes and F4/80+ macrophage or microglial cells (Fig. 4C, D). As observed under light microscopy after luxol fast blue staining, myelin vesiculation and demyelination (Fig. 4G), were present as early as day 11, were maximal between days 14 and 21 and were not detectable after day 28.

In contrast to BALB/c mice, the CNS inflammation in mu/nu and SCID mice was limited to a few small foci of mononuclear cells which were visible in the white matter from day 7. The pathology was studied in detail in SCID mice. The majority of these cells were F4/80+ (Fig. 4F). No Lyt1+ cells were detected (Fig. 4E). From day 11 foci of granule cell pycnosis were observed in the cerebellum (Fig. 4L, M). Both areas containing F4/80+ cells and areas of granule cell pycnosis contained cells positive for viral RNA. Most foci of RNA positive cells showed neither inflammatory cells nor cell death, and although pycnotic nuclei and neuronophagia (Fig. 4J) of cells both positive and negative for viral RNA were occasionally observed, most cells positive for viral RNA appeared morphologically normal (Fig. 4K). There was no perivascular cuffing or demyelination (Fig. 4H) although small cystic lesions were observed in the white matter of the cerebellum at late time points (days 21 to 32). A few of these contained F4/80+ cells but no Lyt1+ cells were observed. On examination at days 14, 21 and 34 the brains of SCID mice given mu/nu or BALB/c sera (d7 or immune serum) or anti-E2 monoclonal had no lesions of demyelination despite, in some cases, persistence of
infectious virus in the brain. Mice sampled at day 10 after d7 spleen cell transfer had no brain virus but had lesions of inflammatory demyelination.

In the skeletal muscles of SCID mice most microscopic fields contained viral RNA positive muscle fibres. Infection was usually confined to single fibres which appeared to be scattered randomly. Most of these infected fibres were morphologically normal (Fig. 4N) with no surrounding inflammatory response. Occasional foci of thinned myofibres with disorganized cytoplasm were observed. These were sometimes surrounded by macrophages (Fig. 4O). Infected muscle fibres were also observed, although rarely, in the skeletal muscles of BALB/c mice on day 2 and these appeared morphologically normal. The lungs of the SCID mice examined at day 32 were congested with thickening of the alveolar walls and blood in the alveoli. Likewise, the kidneys showed congestion of glomeruli. Cells positive for viral RNA could not be observed in either of these tissues. No pathological changes or virus positive cells were observed in liver, kidney, gut, spleen, lymph nodes, pancreas or thyroid tissues of SCID mice at days 28 and 32 or in these tissues in BALB/c or nu/nu mice at days 2 and 4, the time of the viraemia.
Discussion

This report has investigated the course of SFV infection in SCID mice and the role of cell-mediated and humoral immune responses during this infection. Our studies have previously demonstrated that after intraperitoneal infection of 3-4-week or older immunocompetent mice with the avirulent A7(74) strain of SFV a plasma viraemia occurs (Fazakerley et al., 1993). Early studies measuring infectivity and observations by electron-microscopy indicated that SFV A7(74) replicates in skeletal muscle (Pathak et al., 1976; Pusztai et al., 1971). Here we demonstrate for the first time that this involves scattered, single myofibres in skeletal muscle and that small foci of infection are also established in the myocardium. The scattered focal nature of the infection is consistent with random infection from the extracellular fluid and is particularly widespread and easily observed in SCID mice, presumably as a result of the persistent viraemia which is established in the absence of antibodies. Infection of skeletal muscle fibres but not heart muscle was also observed in BALB/c mice. The presence of virus at high levels in the blood of SCID mice for up to 32 days would have been sufficient to circulate infectious virus throughout the body and provide an opportunity for virus to replicate in all permissive cells; however, the only viral RNA positive cells outside the CNS were skeletal muscle myofibres and myocardium.

Once established the viraemia seeds virus into perivascular foci in the CNS where replication of virus in CNS cells is restricted (Pathak et al., 1976; Pathak & Webb, 1978; Fazakerley et al., 1993). The present results support these findings and demonstrate that confinement of the CNS infection to small foci is independent of specific immune responses since this pattern is also observed in infected SCID mice. The increase in the number of foci of CNS infections in SCID mice can be attributed to continued seeding of new perivascular foci from the persistent viraemia. In immunocompetent mice, infection of CNS cells leads to lesions of demyelination as observed here in BALB/c mice. The absence of these lesions in infected nu/nu and SCID mice or after transfer of immune serum to SCID mice and their presence in mice receiving spleen cell transfers confirms our previous findings that demyelination is dependent on T cells, specifically CD8+ T cells (Amor & Webb, 1987; Berger, 1980; Fazakerley et al., 1983; Fazakerley & Webb, 1987b; Subak-Sharpe et al., 1993).

In BALB/c mice, virus was cleared from the blood by day 4 and from the brain by day 7. In contrast, SFV infection of athymic (nu/nu) mice, which lack functional T lymphocytes but synthesize anti-viral IgM, resulted in clearance of the viraemia but not the CNS infection, which persisted for months in small foci scattered throughout the brain and spinal cord. Infection of SCID mice, which lack the ability to produce either antibody or T cells, resulted in a persistent infection of both blood and brain. That IgM is responsible for clearance of viraemia but not brain virus titres is confirmed by the ability of nu/nu serum to reproduce the nu/nu pattern of infection on transfer into infected SCID mice. The failure of this serum to affect the CNS infection may be due to either an inability of IgM to cross the blood-brain barrier or its ineffectiveness within the CNS.

The d7 and immune sera from BALB/c mice, which contain both IgM and IgG, were able, respectively, to reduce and clear infectious brain virus titres. Importantly however, this clearance was not confirmed by in situ hybridization at either day 7 or day 34. A number of possible explanations which independently or in concert could explain this include the following: (i) in situ hybridization is more sensitive than plaque assays; (ii) plaque assays give a false impression of clearance with a significant proportion of neutralization occurring only at the time the brains are homogenized for assay; (iii) the viral RNA positive cells detected do not contain infectious material; or (iv) antibody cannot clear virus from all cells. The last point may be particularly relevant since in similar studies on Sindbis virus infection of SCID mice, after a single dose of hyperimmune serum at day 7 post-infection no infectious virus could be detected in the brain for a month thereafter but that after this time virus was again detectable by infectivity assay (Levine & Griffin, 1992). No in situ hybridization studies to determine presence of virus positive cells in the CNS during this time were undertaken. In both the present study and that on Sindbis virus, the dose of antibody, the isotypes present and the timing and course of antibody administration relative to that of the infection are likely to be important variables. At present, it is difficult to draw any firm conclusions as to whether antibody alone, perhaps if present for a sufficient time can eradicate virus from the brain or whether complete clearance requires T cells. Further studies are in progress. How the non-neutralizing IgG2a monoclonal antibody reduces brain virus titres is not clear. However, it is not unique: this phenomenon has also been described for Sindbis virus where it appears that anti-E2 monoclonal antibodies can mediate clearance of virus from CNS cells, perhaps by restricting viral gene expression (Levine et al., 1991). In addition to alphaviruses, antibodies are important in clearing virus from the CNS or initiating restricted replication in CNS cells in a number of other infections including TMEV, reovirus, rabies virus, mouse hepatitis virus and measles virus infections (Fujinami et al., 1989; Kurtz et al., 1995; Tyler et al., 1989; Perry & Lodmell, 1991; Buchmeier et al., 1984; Liebert et al., 1990).

Despite inoculation (i.p.) of a low virus dose which in...
Fig. 4. For legend see opposite.
BALB/c mice did not induce any clinical signs. SCID mice showed a high rate of paralysis and mortality. Our previous studies have shown that treatment of SFV A7(74) infected mice with immunosuppressive therapies including drugs and 8-0 Gy total body irradiation induces paralysis and mortality whereas infection of athymic nu/nu mice is avirulent (Jagelman et al., 1978; Amor & Webb, 1987; Fazakerley & Webb, 1987a,b). Survival of nu/nu mice can therefore be attributed to their synthesis of anti-viral IgM, which is absent in mice given total body irradiation and in SCID mice. We were unable to ascertain the exact cause of death of infected SCID mice. Pycnosis of cerebellar granule cells, presence of virus in skeletal muscle which could have influenced mobility, viral RNA in the myocardium associated with myocyte destruction and congestion of tissues including the kidney and lungs could each have been fatal or could all have contributed to the animals demise.

Pycnosis of cerebellar granule cells is also observed in SFV infected mice given total body irradiation and other immunosuppressive therapies but does not occur in SFV infected nu/nu mice, despite virus persistence in the CNS (Amor & Webb, 1987; Fazakerley & Webb, 1987a,b). The high brain virus titres seen in the SCID mice probably reflect increased numbers of foci of CNS infection which increase the chance of granule cell infection. It remains unclear whether these cells undergo destruction whenever they are infected, perhaps because they are susceptible to virus triggered programmed cell-death (Levine et al., 1993), and antibody normally prevents spread of infection to or within this cell layer, or whether antibody converts infection of these cells from productive to restricted. It seems likely that antibody has more effect than just decreasing the chance of granule cell infection since we have never observed foci of granule cell pycnosis in immunocompetent adult BALB/c mice, despite infection of adjacent Purkinje cells and cells in white matter tracts. Antibody mediated clearance of alphavirus infection from neurons by restricting viral gene expression has been shown for the closely related Sindbis virus (Levine et al., 1991).

With the exception of the foci of cerebellar granule cell pycnosis, CNS infection in SCID mice was associated with minimal cell destruction. Occasional pycnotic nuclei were observed but most infected cells appeared morphologically normal. CNS infection with minimal destruction was also observed in persistently infected nu/nu mice, where scattered infected cells could be detected for months after infection. Such non-destructive infection of neurons could affect neuronal function as has been observed with lymphocytic choriomeningitis virus (Lipkin et al., 1988). SFV A7(74) infection of immunocompetent mice has been reported to suppress levels of neurotransmitters including noradrenaline and adrenaline (Barrett et al., 1986; Mehta et al., 1993) which are important in controlling behaviour and mood. In the CNS of neonatal and suckling mice, SFV A7(74) infection is productive (Pathak & Webb, 1978; Fazakerley et al., 1993). The present study indicates that the change in age-related virulence is not related to maturity of specific immune responses. That maturity of non-specific immunity such as interferon or NK cell responses determines age-related virulence cannot be ruled out, but developmental changes in CNS cells are likely to be the main factor (K. Oliver & J. K. Fazakerley, unpublished).

In summary, following SFV A7(74) infection virus

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**Fig. 4.** Histopathological changes and localization of SFV RNA by in situ hybridization in the CNS of BALB/c and SCID mice infected with SFV A7(74). (A) Virus infected cell adjacent to a blood vessel (v), BALB/c mouse day 4 post-infection, paraffin section stained with haematoxylin and eosin. (B) Cells positive for viral RNA (arrows) are usually observed within foci of inflammation, BALB/c mouse, day 7, paraffin section, stained with haematoxylin and eosin. (C) Lyt1+ inflammatory cells (arrowed), BALB/c mouse, day 14, frozen section. (D) F4/80+ cells (arrowed), BALB/c mouse, day 14, frozen section. (E) No Lyt1+ cells are present in the CNS of SCID mice, day 14, frozen section. (F) F4/80+ cells (arrowed), SCID mouse, day 14, frozen section. (G) Lesion of demyelination (d), white matter tract of the cerebellum, BALB/c mouse, day 11. The cells of the adjacent granule cell layer (g) are intact. Paraffin section stained with luxol fast blue and cresyl fast violet. (H) White matter (wm) tract of cerebellum, day 11, SCID mouse demonstrating no demyelination, no demyelination and intact granule cell layer (g). Paraffin section stained with luxol fast blue and cresyl fast violet. (I) Lesion of demyelination in the white matter tract of the cerebellum containing cells positive for viral RNA (arrows) by in situ hybridization. BALB/c mouse at 11 days post-infection. Paraffin section stained with haematoxylin and eosin. (J) Neuronophagia of a neuron (n) positive for viral genome by in situ hybridization. Macrophage-like cells (arrows) can be seen to outline the infected cell. SCID mouse, day 11, paraffin section stained with haematoxylin and eosin. (K) In SCID mice most infected CNS cells appear morphologically normal, day 16, paraffin section. Cells in the hypothalamus positive for viral RNA appear morphologically normal. The dark staining cytoplasm of these cells indicates the presence of viral RNA as visualized by in situ hybridization using a digoxigenin labelled riboprobe and detected using the NBT/BCIP reaction. The cytoplasm of the cells is positive, consistent with the known site of replication of this RNA virus. The central nuclei remain unstained. (L) Large focus of granule cell pycnosis in the cerebellum of a SCID mouse, 21 days post-infection. Paraffin section stained with haematoxylin and eosin. (M) Small focus of granule cell pycnosis (arrows). Cerebellum of a SCID mouse 14 days post-infection, paraffin section stained with haematoxylin and eosin. (N) Morphologically normal, SFV RNA positive muscle fibre (mf). SCID mouse, 28 days post-infection. In situ hybridization on a paraffin section using a digoxigenin labelled riboprobe detected with the NBT/BCIP reaction, counterstained with haematoxylin and eosin. (O) Degenerating viral RNA positive (dark staining cytoplasm) muscle fibre with disintegration of cytoplasm, surrounded by macrophage-like cells (arrowed), SCID mouse, day 28, paraffin section. In situ hybridization as in (N).
replicates in skeletal muscle and myocardium generating a viraemia which seeds virus into perivascular foci in the CNS. IgM antibodies clear the viraemia. IgG antibodies, including non-neutralizing antibodies can reduce and even ablate infectivity titres but cells positive for viral RNA remain. T cells (CD8+) are necessary for the development of the lesions of demyelination and the restricted and focal nature of this infection in mature CNS cells is independent of specific immune responses.

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References


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