The type I interferon system protects mice from Semliki Forest virus by preventing widespread virus dissemination in extraneural tissues, but does not mediate the restricted replication of avirulent virus in central nervous system neurons

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Semliki Forest virus (SFV) infection of the mouse provides a powerful model to study the pathogenesis of virus encephalitis. SFV and other alphavirus-based vector systems are increasingly used in biotechnology and medicine. This study analysed the strong susceptibility of this virus to type I interferon (IFN) responses. Following intraperitoneal infection of adult mice, SFV strain A7(74) was efficiently (100 %) neuroinvasive. In contrast, SFV4 was poorly (21 %) neuroinvasive. Upon entry into the brain, both viruses activated type I IFN responses. As determined by quantitative RT-PCR, activation of the IFN-α gene was proportional to virus RNA load. An intact type I IFN system was required for protection against both strains of SFV. IFN strongly curtailed virus spread in many cell types and in many tissues. In mice with an intact type I IFN system, infected cells were rarely observed and tissue tropism was difficult to determine. In the absence of a functional type I IFN system, the tropism and the potential for rapid and widespread infection of this virus was revealed. Virus infection was readily observed in the myocardium, endocardium, exocrine pancreas, adipose tissue, smooth muscle cells and in the brain in meningeal cells, ependymal cells and oligodendrocytes. In the brains of mice with and without type I IFN responses, virus infection of neurons remained rare and focal, indicating that the previously described restricted replication of SFV A7(74) in neurons is not mediated by type I IFN responses.

INTRODUCTION

Semliki Forest virus (SFV) is a mosquito-borne virus that naturally circulates in sub-Saharan Africa. The virus is an alphavirus of the family Togaviridae. Natural human and equine infections have been described (Mathiot et al., 1990). The virus is closely related to Chikungunya virus, responsible recently for an outbreak of severe arthralgia in the islands of the Indian Ocean (Schuffenecker et al., 2006). Other alphaviruses include Sindbis virus in North Africa and Europe; Eastern, Western and Venezuelan equine encephalitis viruses in the Americas and Ross River virus in Australia. SFV infection of laboratory mice provides a tractable model system for the study of virus pathogenesis and in particular virus encephalitis (Fazakerley, 2004). Virulence in mice has been characterized for several natural isolates and their laboratory-passaged strains (Bradish et al., 1971). The most commonly studied strains include A7 and A7(74), which are avirulent in adult mice, and L10 and the prototype, which are virulent in adult mice. All strains of SFV are virulent in neonatal or young suckling mice (Bradish et al., 1971; Pusztai et al., 1971; Seamer et al., 1967).

The most characterized avirulent strain of SFV, A7(74), is virulent in mice infected at the age of 11 days or less, but is avirulent in older mice; virus dissemination in the central nervous system (CNS) is increasingly restricted with age (Oliver et al., 1997). In 4–5-week-old mice, intraperitoneal inoculation of SFV A7(74) results in a high-titre plasma viraemia from which virus is seeded into perivascular foci in the brain and spinal cord; there is little spread of virus
from cell to cell, foci do not enlarge with time and the infection is restricted in mature neurons (Fazakerley et al., 1993). SFV A7(74) remains avirulent following direct intracerebral inoculation, but inoculation by this route results in a widespread infection of oligodendrocytes in the major white matter tracts (Fazakerley et al., 2006). The underlying events resulting in restriction of this strain of the virus in mature neurons remain undetermined. Intraperitoneal infection with the virulent L10 strain also results in a high-titre plasma viraemia, but in this case neuronal infection is not restricted and perivascular foci of CNS infection rapidly enlarge to give rise to a fatal panencephalitis (Fazakerley et al., 1993).

The prototype (SFV4) and A7(74) strains are available as molecular cDNA clones (Liljestrom & Garoff, 1991; Vahakoskela et al., 2003). Infectious RNA can be derived by in vitro transcription and infectious virus by electroporation of this RNA into eukaryotic cells. In mice, SFV4 virus is virulent by intranasal or intracerebral inoculation (Fazakerley et al., 2002; Glasgow et al., 1991). There are numerous genetic changes between the cDNA clones of SFV4 and SFV A7(74), but changes in non-structural protein 3 and the 5′-untranslated sequences appear to be the most important in determining the ability of the virus to replicate in the adult mouse brain (Tuititala et al., 2000). Molecular engineering of the SFV cDNA has led to the generation of a series of replicon vectors that are increasingly used for protein expression, transient gene transfer and increasingly importantly vaccination (Karlsson & Liljestrom, 2004). The vector replicon can be packaged into virus-like particles (VLPs), which have the ability to infect cells in the same way as virus (Smerdou & Liljestrom, 1999). Following infection with virus or VLPs, or following transfection of replicon RNA, cells in continuous culture rapidly undergo apoptosis (Allsopp & Fazakerley, 2000; Glasgow et al., 1997; Scallan et al., 1997).

Not long after the discovery of the interferon (IFN) system, it was shown that a crude preparation of IFN derived from West Nile virus-infected mice could protect mice against the virulent MB strain of SFV (Finter, 1966). In this and subsequent studies, the extent of protection was clearly dependent on virus strain, dose and time of administration (Bradish & Tittmuss, 1981; Smillie et al., 1973). Conversely, administration of anti-IFN antibodies exacerbated SFV infection (Fauconnier, 1971). The kinetics of the type I IFN response in SFV A7(74)-infected mice parallels the plasma viraemia (Bradish et al., 1975). In the original studies on mice with disruption of the type I IFN receptor x-chain (IFNAR-1−/−), it was demonstrated that both adult and neonatal mice without a functional type I IFN system succumbed to infection with SFV much more rapidly than did wild-type (wt) mice (Hwang et al., 1995; Muller et al., 1994). The type I IFN system has also been demonstrated to be crucial for the protection of mice from nominally avirulent strains of the related alphaviruses Venezuelan equine encephalitis virus and Sindbis virus (Grieder & Vogel, 1999; Ryman et al., 2000). Strains of SFV and eastern equine encephalitis virus vary in their sensitivity to IFN (Aguilar et al., 2005; Deuber & Pavlovic, 2007). What remains to be determined for SFV is the role of the type I IFN system following infection with the most commonly used A7(74) and SFV4 strains. Furthermore, not only for alphaviruses but for many neurotropic viruses, the course and role of IFN responses in the brain during virus encephalitis remain to be fully characterized. In the case of SFV encephalitis, it remains to be determined whether the type I IFN system is involved in the restricted replication of A7(74) in neurons and in the age-related virulence (Fazakerley et al., 1993). Here, we have shown that: (i) both A7(74) and SFV4 activate CNS type I IFN gene expression in the mature mouse brain; (ii) IFN gene expression is proportional to virus load; (iii) the type I IFN response is crucial for protection against SFV A7(74); and (iv) this protection works by strongly curtailing virus spread in many cell types in many tissues including some CNS cell types but is not responsible for the restricted dissemination of SFV A7(74) in mature CNS neurons.

METHODS

Viruses. The A7(74) strain of SFV was derived from the AR2066 strain by seven passages through neonatal mouse brain and colony selection on chick embryo fibroblasts; AR2066 was isolated from Aedes argenteopunctatus mosquitoes in Namancurra, Mozambique (McIntosh et al., 1961). The L10 strain was derived from the original isolate of SFV (Smithburn & Haddow, 1944) by eight intracerebral passages through adult and two through baby mouse brains (Bradish et al., 1971). Infectious SFV4 virus was generated from a cDNA plasmid derived from the prototype strain of SFV (Liljestrom & Garoff, 1991). The full-length pSP6-SFV4 cDNA was a kind gift from Professor Peter Liljestrom, (Karolinska Institute, Stockholm, Sweden).

SFV4 marker virus containing the gene for enhanced green fluorescent protein (eGFP) was constructed by inserting the coding sequence for egFP followed by the foot-and-mouth disease virus 2A cleavage sequence between that for the capsid protein and p62 in the virus structural protein open reading frame. This strategy has been used previously to construct an eGFP-labelled Sindbis virus (Thomas et al., 2003). The resulting SFV4 marker virus, designated SFV4-steGFP, replicated well in vitro and in vivo, and infected cells showed strong green fluorescence (R. Fragkoudis and others, unpublished). SFV4 and SFV4-steGFP were generated from infectious cDNAs as described previously (Liljestrom & Garoff, 1991). Briefly, the plasmid was linearized with Sphi and capped transcripts were produced by in vitro transcription with SP6 polymerase in the presence of m′GppG cap analogue (Amersham). RNA was electroporated into BHK-21 cells using two consecutive 140 V square-wave pulses with a pulse length of 25 ms on a Bio-Rad Gene Pulser X cell electroporator. Virus stocks were collected after 24 h at 37 °C, titrated on monolayers of BHK-21 cells and used to infect mice.

Virus infectivity assay. Virus titres were determined by plaque assay on BHK-21 cells as described previously (Fazakerley et al., 1993). Briefly, BHK cells (4 × 10⁵) were seeded into six-well plates in 2 ml Glasgow minimum essential medium with 10% fetal calf serum and incubated at 5% CO₂ and 37 °C until 80% confluent. The growth medium was then removed, cells washed with PBS and 10-fold dilutions of test samples were titrated in duplicate. Infected cells were incubated for 1 h at room temperature in a humid atmosphere. After
the addition of agar, the plates were incubated for a further 48 h and fixed with 10% neutral buffered formaldehyde for 3 h before staining with 0.1% toluidine blue for at least 1 h. Plaques were counted after washing.

Mouse strains. Strain 129/Sv(ev) and 129/Sv(ev) mice with a disruption of the α-chain of the IFN-α/β receptor (IFNAR-1-/-) (Muller et al., 1994) were purchased from B&K Universal Ltd. BALB/c and CB17 nu/nu mice were purchased from Harlan Olac. Mice were bred and maintained in the Centre for Infectious Diseases Animal Unit, College of Medicine & Veterinary Medicine, University of Edinburgh, UK, in specific-pathogen-free and environmentally enriched conditions with 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 licence.

Infection of mice. Experimental infection of mice was carried out at 4–6 weeks of age. Mice were inoculated intraperitoneally with 0.1 ml PBS with 0.75% BSA (PBSA) containing 5×10^5 p.f.u. SFV or intracranially with 20 μl PBSA containing 10^5 p.f.u. virus or with PBSA alone. Mice were checked twice daily and euthanized on reaching previously defined clinical endpoints considered to be indicative of fatal disease; these included: paralysis in two or more limbs; inability to move, feed or drink; incontinence; breathing difficulties; and seizures. Mice to be sampled were deeply anaesthetized with halothane and perfused with PBS through the left cardiac ventricle; brains were removed, divided bilaterally down the midline and either placed in RNAlater for RNA analysis, snap frozen on dry ice for virus titration or immersion fixed in 10% phosphate-buffered formal saline for histopathological study.

RNA extraction and quantitative PCR. RNA was extracted from frozen cell-culture pellets or tissue samples stored in RNAlater using Qiagen kits according to the manufacturer’s instructions; the RNeasy Lipid kit was used for brain samples. Extracted RNA was stored at -80°C. The quality of the extracted RNA was determined using an Agilent 2100 Bioanalyzer and the RNA 6000 Nano assay. High-quality RNA samples were reverse transcribed using Superscript II RNase H reverse transcriptase. Samples to be directly compared were reverse transcribed at the same time using the same master mix (all reagents from Invitrogen). The reaction mixture had a final volume of 20 μl and contained 1 μl oligo(dT)12-18 primer, 5 μg RNA template, 1 μl 10 μM dNTPs and DNase/RNase-free water to a volume of 12 μl. The mixture was heated for 5 min at 65°C. Next, 4 μl 5× First-strand buffer, 2 μl 0.1 M DTT and 1 μl RNasin recombinant RNase inhibitor were added. Reactions were incubated at 42°C for 2 min and 1 μl reverse transcriptase was added. The reactions were incubated at 42°C for 1 h. The enzyme was inactivated by incubation for 15 min at 70°C. The cDNA was stored at -20°C.

Levels of viral RNA and IFN transcripts were determined by real-time quantitative (Q)PCR. Test samples and standards were assayed in triplicate. QPCR was performed using a FastStart DNA Master SYBR Green I kit (Roche). Briefly, in a total volume of 20 μl (made up in RNase-free water), reaction mixes included: 50 pmol primers, 40 μM dNTPs, 10× buffer plus 2 mM MgCl₂, SYBR Green (diluted 1:20 000; Biogene), 5 U FastStart Taq (Roche Applied Science) and 2 μl cDNA. Tubes were heated to 94°C for 5 min and 40 cycles of 94°C for 20 s, 62°C for 20 s and 72°C for 20 s were carried out on a RotorGene 3000 (Corbett Research). As a positive control for SFV, an in vitro transcript from the pGEM1-SFV cDNA plasmid containing the structural genes of SFV was transcribed using a Promega RiboMax kit. Serial dilutions of this plasmid were used to produce a standard curve for quantification of the PCR amplicons and thus the virus RNA. Brain samples were normalized to levels of β-actin transcripts (Brown et al., 2003). Sequences of the primers used in the assay were as follows: IFN-α: 5'-AGCACAGGAAGATTTTGGA-3' and 5'-GGCTGCTATGGGATCTT-3' (degenerate primers based on IFN-α/β, McKimmie & Fazakerley, 2005); SFV E1: 5'-CGGACTACCTTCTTGTG-3' and 5'-CCAGACACCCCGATTTT-3'; IFN-β: 5'-GACAGCCCTTCTCATCAA-3' and 5'-GACTTCCTCGCTCATTCC-3'; β-actin: 5'-CGTGCATCTCGTAAAGACC-3' and 5'-CTGGAAGGTGACAGTGG-3'.

In situ hybridization. To observe virus distribution in the brain, after immersion fixation in 10% phosphate-buffered formal saline, half brains were embedded in paraffin and 5 μm sections cut onto poly-l-lysine-coated (Sigma) or Biobond-coated (British BioCell International) glass slides. Riboprobes were transcribed in vitro with T7 polymerase (using a Riboprobe Gemini kit; Promega) from HinClI-linearized pGEM1-SFV. As a control, some sections were hybridized with a riboprobe to the P1 region of the unrelated Theiler’s virus. Probes were hydrolysed in 0.04 M NaHCO₃ for 30 min at 60°C prior to use. In situ hybridization with 35S-labelled riboprobes was carried out as described previously (Fazakerley et al., 1993). Autoradiographic images of sections hybridized with 35S-labelled riboprobes were produced by exposure of air-dried sections to Hyperfilm βmax (Amersham). Sections were subsequently dipped in photographic emulsion (LM-1, diluted with 0.66 M ammonium acetate; Amersham) and exposed, usually for 7 days, at 4°C.

Histopathology. Immunostaining to detect SFV-infected cells in paraffin-embedded tissues sections was performed as described previously (Fazakerley et al., 2006). Tissues to be studied for the distribution of eGFP-positive cells were fixed in 10% phosphate-buffered formal saline overnight and examined on a Zeiss fluorescent stereomicroscope with a GFP filter before cryopreserving by sequential passage through 5, 10 and 25% sucrose in PBS. After freezing in OCT, 12–14 μm sections were cut onto poly-l-lysine-coated glass slides using a cryomicrotome. Sections were stained with propidium iodide to visualize cell nuclei or were lightly stained with diaminobenzidine to allow visualization of the tissue structure (this preserved the eGFP signal better than conventional stains such as haematoxylin).

RESULTS

SFV A7(74) but not SFV4 is efficiently neuroinvasive in adult mice, and brain infection induces type I IFN gene expression

Following intraperitoneal inoculation, SFV A7(74) has previously been demonstrated to be efficiently and consistently neuroinvasive (Fazakerley et al., 1993; Pusztau et al., 1971). SFV4 is neuroinvasive if given intranasally (Glasgow et al., 1991). The neuroinvasive efficiency of SFV4 following intraperitoneal infection has not been studied in detail. To determine whether SFV4 is naturally neuroinvasive following intraperitoneal inoculation and to determine whether SFV neuroinvasion activates the type I IFN system, 4–5-week-old BALB/c mice were inoculated intraperitoneally with 5000 p.f.u. SFV A7(74) or SFV4. At days 3 and 7 post-inoculation (p.i.), five mice from each group were exsanguinated under terminal anaesthesia, perfused through the left cardiac ventricle with PBS to remove any remaining blood in the circulatory system, and the brains removed and assayed for infectious virus and IFN gene transcripts. Levels of infectious virus were assayed...
by standard plaque assay. Levels of IFN transcripts were assayed by QPCR. QPCR was used in preference to assaying functional IFN as RNA levels are less likely to be affected by levels of blood-derived material, particularly as SFV A7(74) is known to disrupt the integrity of the blood–brain barrier (Parsons & Webb, 1982).

At 3 and 7 days p.i., all (10/10) mice infected with SFV A7(74) had infectious virus and IFN-α gene transcripts detectable in the brain (Table 1), demonstrating that the type I IFN system was activated in response to neuroinvasion by this strain of the virus. The levels of infectious virus and the induced levels of IFN-α transcripts showed considerable variation between individual mice. For the virus titres, this was consistent with previous studies (Fazakerley et al., 1993). Presumably, variation resulted from temporal and spatial differences in virus neuroinvasion among individual mice. Interestingly, for individual mice there was a very strong correlation ($r^2=0.98$) between levels of virus RNA and levels of IFN transcripts (Fig. 1a); the higher the brain virus titre, the higher the level of IFN transcripts. Presumably, the greater the number of virus-infected cells, the greater the number of cells producing IFN.

In contrast to SFV A7(74) infection, intraperitoneal infection with SFV4 resulted in only one of the five mice at day 3 and none of the five mice at day 7 p.i. having detectable infectious brain virus. Intraperitoneal infection with SFV4 was repeated with nine mice per group with similar results; only two had infectious virus in the brain at day 3 and only one had infectious virus at day 7 p.i. In this second experiment, the mean titre of the three virus-positive brains was 2.3 $\log_{10}$ p.f.u. g$^{-1}$. It was concluded that SFV4 is poorly neuroinvasive, with only 21 % of mice (3/14) demonstrating neuroinvasion at day 3 p.i.

**Direct intracerebral inoculation of SFV4 results in widespread brain infection and activation of IFN gene expression**

Following intracerebral inoculation, SFV4 replicates efficiently in the CNS and produces a widespread infection (Fazakerley et al., 2002). To determine whether this infection activates IFN gene transcription, SFV4 was inoculated intracerebrally into six 4–5-week-old BALB/c mice. Two mice were each sampled at 12, 24 and 40 h p.i. Infectious brain virus titres were detectable in all mice at all

### Table 1. Levels of infectious virus and IFN-α transcripts in the brains of BALB/c and CB17 nu/nu mice at 3, 7 and 140 days p.i. following intraperitoneal inoculation with 5000 p.f.u. SFV A7(74)

Results are the mean values from at least four individual mouse brains. Virus titres were determined by standard plaque assay on BHK-21 cells and are given as $\log_{10}$ p.f.u. (g brain)$^{-1}$. IFN-α gene transcripts were measured by QPCR and are given as the mean fold increase (MFI) relative to uninfected brain tissue (which was given a value of 1 and was effectively zero, the limit of detection of the assay).

<table>
<thead>
<tr>
<th>Days p.i.</th>
<th>BALB/c mice</th>
<th>CB17 nu/nu mice</th>
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<tr>
<td></td>
<td>No. of mice positive for virus</td>
<td>Brain virus $\log_{10}$ p.f.u. g$^{-1}$</td>
</tr>
<tr>
<td>3</td>
<td>5/5</td>
<td>2.8</td>
</tr>
<tr>
<td>7</td>
<td>5/5</td>
<td>3.9</td>
</tr>
<tr>
<td>140</td>
<td>0/5</td>
<td>&lt;1.4</td>
</tr>
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time points. IFN-α gene transcripts were detectable in three out of six mice, with a mean titre of 10^{2-9} copies per 10^5 copies of β-actin (Fig. 1b). SFV4 thus had no deficit in its ability to replicate in the brain and this replication activated IFN-α gene expression.

**IFN gene transcription in the brain continues during persistent infection**

In mice with severe combined immunodeficiency (SCID) or athymic nu/nu mice, SFV A7(74) establishes a persistent infection of the brain; athymic nu/nu mice survive this infection longer than SCID mice (Amor & Webb, 1986; Fazakerley & Webb, 1987). To determine whether the brain IFN response remained activated during persistent infection, CB17 nu/nu mice were inoculated intraperitoneally with 5000 p.f.u. SFV A7(74), exsanguinated, perfused and the brains assayed at 3, 7 and 140 days p.i. Infectious virus and IFN transcripts were detectable in all mice at all three time points (Table 1).

**Type I IFN is required for protection against SFV A7(74)**

To determine whether the type I IFN system is required to protect adult mice against intraperitoneal infection with SFV, groups of ten 129/Sv(ev) mice lacking the x-chain of the IFN-α/β receptor (IFNAR-1<sup>-/-</sup>) (Muller et al., 1994) and IFN-competent 129/Sv(ev) mice were inoculated intraperitoneally with 5000 p.f.u. SFV A7(74) or SFV4 and monitored for clinical disease. All 129/Sv(ev) mice inoculated with SFV4 or A7(74) survived without clinical signs for the 10 days studied. SFV4 virus is transcribed from a molecular clone derived from the SFV prototype virus, a virus closely related to SFV L10. Both prototype and L10 are virulent, even at low doses, in adult mice (Bradish et al., 1971). It was surprising, therefore, that inoculation of 5000 p.f.u. SFV4 intraperitoneally was avirulent in 129/Sv(ev) mice. For comparison, a group of ten 129/Sv(ev) mice were inoculated intraperitoneally with 5000 p.f.u. SFV L10 virus. These mice all died or reached clinically defined terminal end points on day 3. It was concluded that, relative to virulent L10 virus, SFV4 has an attenuated phenotype when given intraperitoneally. In contrast to the survival of 129/Sv(ev) mice, all IFNAR-1<sup>-/-</sup> mice inoculated with SFV A7(74) or SFV4 died or reached clinically defined terminal end points within 3 days. The fact that SFV4 and SFV A7(74) are rapidly virulent in IFNAR-1<sup>-/-</sup> mice but not in IFN-competent mice demonstrated that type I IFN strongly and successfully suppresses these strains of this virus.

**Absence of a type I IFN response demonstrates the tissues and cell types permissive for SFV infection**

Given that mortality in the IFNAR-1<sup>-/-</sup> mice was not related to widespread CNS infection, the course of extraneuronal infection was determined. Groups of 129/Sv(ev) and IFNAR-1<sup>-/-</sup> mice were inoculated intraperitoneally with 5000 p.f.u. SFV A7(74) or SFV4 with an eGFP marker gene inserted into the structural protein open reading frame (SFV4-steGFP). Three mice were sampled from each group of mice at days 1 and 2 p.i. Blood samples were taken for virus titration and samples of tissues from several different organ systems were studied by microscopy. Tissues from the SFV A7(74)-infected mice were processed for paraffin histology, cut into 5 μm sections and immunostained to visualize virus-infected cells (Fig. 3). Tissues from SFV4-steGFP-infected mice were fixed, cut
into 12–14 μm sections on a cryomicrotome and studied on a Zeiss confocal microscope (Fig. 4). At 1 day p.i., the mean \( n=3 \) blood virus titres were \( 10^{9.2} \) and \( 10^{8.5} \) log_{10} p.f.u. ml\(^{-1}\) in IFNAR-1\(^{-/-}\) mice and \( 10^{7.8} \) and \( 10^{4.4} \) log_{10} p.f.u. ml\(^{-1}\) in 129/Sv(ev) mice for SFV A7(74) and SFV4, respectively. In SFV-infected 129/Sv(ev) mice, despite examination of many sections from many tissues including brain, lung, liver, kidney, heart, spleen, small intestine, pancreas, skeletal and smooth muscle, virus-infected cells were observed only rarely, most frequently in the exocrine pancreas. In stark contrast, infected cells were readily observed in all of these tissues in IFNAR-1\(^{-/-}\) mice, demonstrating the power of the type I IFN system to curtail virus infection. No differences in tropism were observed between the two viruses in IFNAR-1\(^{-/-}\) mice. Most tissues studied showed a selective cellular tropism (Figs 3 and 4 and Table 2). For example, cells of the exocrine but not the endocrine pancreas were heavily infected; many cells in the heart valves but only relatively few cells in the myocardium were infected; many cells of the marginal zone of the spleen, most probably macrophages, were infected, as were lymphocytes in the white pulp; adipose tissue showed the most extensive infection; other infected cells included hepatocytes, smooth muscle cells, cells of the kidney glomeruli and cells in the lung. The difference in the extent of infection between mice with and without a type I IFN system dramatically illustrated the power of this system to limit the spread of this virus. In the absence of

**Fig. 2.** Representative autoradiographic images illustrating the distribution of virus RNA-positive cells (black) in parasagittal sections of the brains of wt 129/Sv(ev) or IFNAR-1\(^{-/-}\) mice 16 or 40 h after intraperitoneal or intracerebral inoculation of SFV A7(74). *In situ* hybridization using a negative-sense \(^{35}\)S-labelled riboprobe complementary to the viral genomic RNA. For each of three mice sampled at each time point, three sections from each of three areas of the brain were studied; the images shown are representative of each group of mice. All brains are in the same orientation; the main olfactory bulb (mob) and the cerebellum (cb) demonstrate the rostro-caudal orientation. Following intraperitoneal inoculation, no virus-positive cells were observed at 16 h and only rare virus-positive cells were observed at 40 h (arrows); the mice sampled at 40 h had clear signs of infection including a hunched posture, piloerection, weight loss, inactivity and tremor. Following intracerebral inoculation in both 129/ Sv(ev) and IFNAR-1\(^{-/-}\) mice, virus-infected cells were present in the major white matter tracts of the corpus callosum and the internal capsule (arrows). In addition in IFNAR-1\(^{-/-}\) mice, ependymal cells surrounding the third and fourth ventricles (3V and 4V, respectively) and meningeal cells (m) were also positive for virus (see also Fig. 3a–c).
an intact IFN system, the true tropism and the potential of this virus for rapid and widespread infection were revealed.

**DISCUSSION**

Previous studies in the early days of IFN research showed that intraperitoneal inoculation of adult mice with SFV A7(74) induced IFN activity in the blood and that crude preparations of IFN protected mice against virulent strains of SFV (Bradish & Titmuss, 1981; Bradish et al., 1975; Finter, 1966; Smillie et al., 1973). The first description of IFNAR-1−/− mice noted that SFV was rapidly fatal in these mice but the strain of SFV used was not stated (Muller et al., 1994). Here, we have shown that both the nominally avirulent SFV A7(74) and the increasingly used SFIV are both virulent in IFNAR-1−/− mice and that, in mice with an intact type I IFN system, both viruses induce IFN gene expression in the brain with levels of IFN-α gene transcripts...
directly proportional to those of viral RNA: the more viral RNA, the more IFN transcripts. Other studies have shown that the type I IFN system is activated in the brain in response to inoculation of poly I:C and to infection with viruses including rabies virus, Theiler’s virus, La Crosse virus, simian immunodeficiency virus and lymphocytic choriomeningitis virus (Barber et al., 2004; Delhaye et al., 2006; Johnson et al., 2006; Prehaud et al., 2005; Roberts et al., 2004). In the present study, our novel QPCR approach demonstrated for the first time that the IFN gene
expression response is proportional to the viral RNA load. In culture, CNS cells including neurons and glial cells have been observed to activate the type I IFN system (McKimmie & Fazakerley, 2005; Prehaud et al., 2005). Intrathecal synthesis of IFNs and IFN-activated protein expression have also been demonstrated in patients with CNS virus infections (Dussaix et al., 1985; Ogata et al., 2004).

The type I IFN system has been shown to protect mice against the spread of other viruses in the CNS including Theiler’s virus, Bunyamwera virus, Dugbe virus, Hantaan virus, influenza A virus, vesicular stomatitis virus, lymphocytic choriomeningitis virus, Sindbis virus and Venezuelan equine encephalitis virus (Boyd et al., 2006; Bridgen et al., 2001; Fiette et al., 1995; Garcia-Sastre et al., 1998; Grieder & Vogel, 1999; Koerner et al., 2007; Muller et al., 1994; Ryman et al., 2000; Wichmann et al., 2002). Few studies have detailed the CNS cell types protected by this IFN response. As documented previously, SFV A7(74) is efficiently neuroinvasive, but in the adult mouse brain it is restricted in its ability to replicate in and spread between mature neurons (Fazakerley et al., 1993, 2006; Oliver & Fazakerley, 1998; Puszta et al., 1971). Mouse neurons, both in culture and in the adult mouse brain, can respond to IFNs (Ousman et al., 2005; Wang & Campbell, 2005; Wang et al., 2002; Ward & Massa, 1995). This response could underlie the restricted replication of SFV A7(74) in neurons. Here, however, we showed that this restricted replication is not mediated by type I IFN responses; A7(74) replication in mature neurons remained restricted, even in the absence of type I IFN responses. Type I IFN responses did, however, control replication of SFV A7(74) in meningeal and ependymal cells, as these cells were infected only rarely in wt mice. Type I IFN responses have also been shown to protect ependymal cells from measles virus, meningeal cells from Sindbis virus and oligodendrocyte, ependymal and choroid plexus cells from Theler’s virus infections (Fiette et al., 1995; Mrkic et al., 1998; Ryman et al., 2000). Given that, in the absence of type I IFN responses, SFV A7(74) does not establish a widespread infection of the brain parenchyma, other factors must be operating to restrict replication of this virus in mature

**Table 2.** Cell types infected by SFV A7(74) and SFV4 in IFNAR-1−/− mice

SFV A7(74)-infected cells were determined by immunostaining and SFV4-steGFP-infected cells were detected by fluorescence. Cell types were determined by morphology and location within tissues. The same cell types were found to be infected by SFV A7(74) and SFV4.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Cell types infected</th>
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<tbody>
<tr>
<td>Brain</td>
<td>Neurons, oligodendrocytes, ependymal cells, meningeal cells but not astrocytes</td>
</tr>
<tr>
<td>Heart</td>
<td>Cells in the valves and occasional cells in the myocardium</td>
</tr>
<tr>
<td>Lung</td>
<td>Alveolar lining cells</td>
</tr>
<tr>
<td>Liver</td>
<td>Hepatocytes and occasional Kupffer cells</td>
</tr>
<tr>
<td>Pancreas</td>
<td>Acinar cells in the exocrine pancreas but not cells in the endocrine pancreas</td>
</tr>
<tr>
<td>Kidney</td>
<td>Cells in the glomeruli and tubule cells</td>
</tr>
<tr>
<td>Adipose</td>
<td>Widespread infection</td>
</tr>
<tr>
<td>Muscle</td>
<td>Occasional skeletal muscle fibres and particularly smooth muscle</td>
</tr>
<tr>
<td>Spleen</td>
<td>Cells in the marginal zone (macrophages?)</td>
</tr>
</tbody>
</table>

**Fig. 4.** Distribution of SFV4 in selected tissues of IFNAR-1−/− mice as visualized by expression (green) of an eGFP marker gene expressed as part of the virus structural polyprotein. For tissue sections (b)–(l), the images are representative of at least nine sections from at least three different areas of each tissue type from at least two mice. In many tissues, areas of infection were apparent, even upon gross examination, as shown for the brain in (a) where areas of infection are present in the inferior colliculi (ic), the frontal cortex (fctx) and the main olfactory bulb (mob); the cerebellum (cb) is marked to facilitate orientation. Some foci of infection appeared to be associated with blood vessels (examples indicated by arrows); however, this was difficult to determine without knowing the depth of tissue imaged. Sections from this brain demonstrated infection of meningeal cells (b; cf. Fig. 2) and several neuronal populations as shown, for example in (c) for pyramidal neurons in the hippocampus (p) and cells in the hilus of the dentate gyrus (dg); red propidium iodide staining of cell nuclei allowed visualization of the neuroanatomy. The sections shown in (d)–(k) were lightly stained with diamobenbenzidine to allow visualization of the tissue structure; red blood cells appeared black. In the heart (d–f), strikingly there was consistent infection of cells in the heart valves (d, e) but only small foci of infected cells in the myocardium (f). In the spleen (g, h), infected cells were principally in the marginal zones (m2) between the red pulp (rp) and white pulp (wp), areas known to contain many macrophages and dendritic cells. In the pancreas (i), many isolated scattered acinar cells were infected (example indicated by arrow) in the exocrine pancreas; infection of cells of the endocrine pancreas was never observed. In the kidney (kd) (j), many cell types including cells in the glomeruli and tubule cells were infected; the smooth muscle cell layers surrounding the ureter (ur) were heavily infected. One of the most widely infected cell types was adipose tissue (k). Foci of infection were apparent in the lung (l). Bars, 100 μm. A high-resolution PDF file of this figure is available as supplementary material in JGV Online.
neurons. We have previously suggested that the restricted replication of A7(74) in the mature neurons of the adult mouse brain is linked to neuronal differentiation and, in particular, to the availability of freshly synthesized intracellular membranes and their constituent lipids (Oliver & Fazakerley, 1998; Oliver et al., 1997; Scallan & Fazakerley, 1999).

We have shown that, although type I IFN does not contribute to the restricted phenotype of SFV A7(74) in adult brain neurons, it is essential to prevent virus spread in extraneural tissues. Several other viruses, including avirulent strains of the related alphavirus Sindbis virus, which, like SFV A7(74), produces subclinical or even unapparent infection, also establish widespread infections in IFNAR-1−/− mice (Bray, 2001; Mrkić et al., 1998; Muller et al., 1994; Ryman et al., 2000). In the case of SFV4 and SFV A7(74), infection was observed in multiple tissues with a similar extent of infection and tropism for both viruses. Whether SFV tropism is determined by entry or post-entry events is unclear. The receptor for this virus and its cellular distribution remain unknown. In the absence of an IFN response, the cell types with the greatest degree of infection were the acinar cells of the exocrine pancreas and adipocytes. To enable their secretion of large amounts of digestive enzymes, pancreatic acinar cells contain large amounts of rough endoplasmic reticulum and have a highly vesiculated cytoplasm. Similarly, adipocytes contain many vacuoles and have a highly developed lipid metabolism. Extensive replication of SFV A7(74) in these cell types would be consistent with a requirement for lipids and freshly synthesized membranes, as suggested for neurons. In the absence of the IFN response, infection of heart valves was consistently observed and was more extensive than infection of other parts of the heart. This could have resulted from a greater exposure of the valves to the high titres of virus in the blood or alternatively from an increased susceptibility of this tissue to infection. In mice, enteroviruses have been shown to preferentially infect valve tissues and some patients with chronic rheumatic heart disease have evidence of enterovirus replication in valve tissue (Burch et al., 1999; Liljestrom, Fazakerley, & McInerney, 2007). Infection was observed in multiple tissues with a similar extent of infection and tropism for both viruses. Whether SFV tropism is determined by entry or post-entry events is unclear.

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In the spleens of IFNAR-1−/− mice infected with Sindbis virus, the distribution of virus infection was observed in multiple tissues with a similar extent of infection and tropism for both viruses. Whether SFV tropism is determined by entry or post-entry events is unclear. The receptor for this virus and its cellular distribution remain unknown. In the absence of an IFN response, the cell types with the greatest degree of infection were the acinar cells of the exocrine pancreas and adipocytes. To enable their secretion of large amounts of digestive enzymes, pancreatic acinar cells contain large amounts of rough endoplasmic reticulum and have a highly vesiculated cytoplasm. Similarly, adipocytes contain many vacuoles and have a highly developed lipid metabolism. Extensive replication of SFV A7(74) in these cell types would be consistent with a requirement for lipids and freshly synthesized membranes, as suggested for neurons. In the absence of the IFN response, infection of heart valves was consistently observed and was more extensive than infection of other parts of the heart. This could have resulted from a greater exposure of the valves to the high titres of virus in the blood or alternatively from an increased susceptibility of this tissue to infection. In mice, enteroviruses have been shown to preferentially infect valve tissues and some patients with chronic rheumatic heart disease have evidence of enterovirus replication in valve tissue (Burch et al., 1999; Liljestrom, Fazakerley, & McInerney, 2007). Infection was observed in multiple tissues with a similar extent of infection and tropism for both viruses. Whether SFV tropism is determined by entry or post-entry events is unclear.

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REFERENCES


