Review

Molecular determinants of alphavirus neuropathogenesis in mice
Gregory J. Atkins¹ and Brian J. Sheahan²

¹Department of Microbiology, Moyne Institute, Trinity College, Dublin 2, Ireland
²School of Veterinary Medicine, University College Dublin, Belfield, Dublin 4, Ireland

Alphaviruses are enveloped viruses with a positive-stranded RNA genome, of the family Togaviridae. In mammals and birds they are mosquito-transmitted and are of veterinary and medical importance. They cause primarily two types of disease: encephalitis and polyarthritis. Here we review attempts to understand the molecular basis of encephalitis and virulence for the central nervous system (CNS) in mouse models. Sindbis virus (SINV) was the first virus to be studied in this way. Other viruses analysed are Semliki Forest virus (SFV), Venezuelan equine encephalitis virus, Eastern equine encephalitis virus and Western equine encephalitis virus. Neurovirulence was found to be associated with damage to neurons in the CNS. It mapped mainly to the E2 region of the genome, and to the nsP3 gene. Also, avirulent natural isolates of both SINV and SFV have been found to have more rapid cleavage of nonstructural proteins due to mutations in the nsP1–nsP2 cleavage site. Immune-mediated demyelination for avirulent SFV has been shown to be associated with infection of oligodendrocytes. For Chikungunya virus, an emerging alphavirus that uncommonly causes encephalitis, analysis of the molecular basis of CNS pathogenicity is beginning. Experiments on SINV and SFV have indicated that virulence may be related to the resistance of virulent virus to interferon action. Although the E2 protein may be involved in tropism for neurons and passage across the blood-brain barrier, the role of the nsP3 protein during infection of neurons is unknown. More information in these areas may help to further explain the neurovirulence of alphaviruses.

Introduction

Alphaviruses are positive single-stranded RNA viruses of the family Togaviridae. Except for two fish viruses, and Eilat virus which only infects insects, alphaviruses infect a range of birds and mammals including humans, and are transmitted by mosquitoes. There are about 30 members of the family, many of which are pathogenic for humans and animals. Chikungunya virus (CHIKV) has recently become of increased importance due to epidemics in Africa, Asia and Indian Ocean islands. Although this virus causes primarily arthralgia in humans, several other members of the genus cause encephalitis in the infected host, and neurological involvement in CHIKV infection has been described (Arpino et al., 2009).

Alphaviruses are enveloped viruses, and infection is initiated by adsorption onto infected cells by the envelope spike proteins. The virus then enters the cell by endocytosis. The mechanism of viral RNA synthesis and expression has been reviewed by Rupp et al. (2015). Briefly, the genome is divided into nonstructural and structural regions that code for the four nonstructural proteins, and the one capsid and three envelope proteins. The structural protein region also codes for a small protein designated 6k, which encompasses the TransFrame protein. There is a gene amplification mechanism whereby the structural proteins are encoded by a subgenomic RNA species that is formed by transcription from an internal promoter on the negative-stranded genomic RNA.

Mouse models of alphavirus inflammation, including in the central nervous system (CNS), have recently been reviewed by Taylor et al. (2015), so will not be described here. Here we will review progress that has been made using mouse models in investigating mechanisms that determine alphavirus neuropathogenesis. We intend to describe published work on Sindbis virus (SINV) and Semliki Forest virus (SFV), two well-known model systems, as well as work on Venezuelan equine encephalomyelitis virus (VEEV), Eastern equine encephalomyelitis virus (EEEV) and Western equine encephalomyelitis virus (WEEV), viruses of medical, as well as veterinary, significance. We will also discuss preliminary work on CHIKV.

Sindbis virus

SINV has been designated the prototype alphavirus because its molecular biology and pathogenesis have been
intensively studied. Indeed SINV was the first alphavirus whose pathogenicity was analysed at the molecular level.

**Mapping of virulence determinants.** In experimental studies, SINV is usually given intracerebrally (i.c.) to mice and multiplies primarily in neurons in the CNS. It has been found that virulence for mice is age dependent. Wild-type SINV is virulent only for neonatal mice, but avirulent for weanling mice. Initially, a neuroadapted strain was produced by passage in mouse brain, which was neuroviralent for weanling mice. In these studies, a full-length cDNA clone of wild-type SINV was utilized, from which infectious RNA could be transcribed. Hybrid cDNA clones were then constructed by replacing restriction fragments in this clone with the corresponding fragments in the strains to be examined, then measuring virulence and sequencing. In this way, the influence of each amino acid change between strains that differed in virulence for neonatal and weanling mice was examined. It was found that for neonatal mice a gradient of virulence existed involving amino acids in the E1 and E2 proteins (Tucker et al., 1993). For the neuroadapted strain, recombinant virus containing just the envelope protein region from the virulent strain was virulent for weanling mice. The determination of neurovirulence was further narrowed to three amino acid changes in the E2 and E1 proteins (Gln-55 to His in E2, Val-72 to Ala and Ala-313 to Gly in E1; Lustig et al., 1988). It was found that E2 His-55 plays an important role, possibly due to binding to neural cells (Lee et al., 2002). It was found that virus with His-55 established infection more efficiently, replicated faster and had a higher rate of release in neuroblastoma cells than virus with Gln at this position. The multiplication rate of the two virus strains in standard BHK cells was similar. Further analysis also showed that E2 Arg-172 to Gly is important for adsorption and penetration, and contributes to neurovirulence (Tucker et al., 1997; Dropulic et al., 1997). For neonatal mice it has been shown that substitution of amino acid Arg-114 for Ser in the E2 protein is associated with attenuation (Polo et al., 1988).

In a separate study, SINV was passaged in mouse brain to increase neuroinvasiveness and neurovirulence. Two isolates that had undergone extensive mouse brain passaging were neurotropic and one caused lethal encephalitis, followed by peripheral inoculation. Virus recovered from infectious clones of these isolates was shown to retain its phenotype in primary cells (Lee et al., 1997). The attenuated virus established infection in the CNS as efficiently as the virulent virus but failed to grow efficiently in the CNS at lat times after infection, suggesting that it either failed to spread or was cleared more efficiently (Heise et al., 2000).

Using an avirulent virus with a double amino acid change in the E2 region (His-55 to Gln, Lys-70 to Glu), Zhu et al. (2015) have shown that the virus reverts to full virulence for adult mice on mutation in the nsP1 region (Thr-173 to Ile). This neurovirulence may be related to the increased efficiency and persistence of propagation of the virus, although the exact nature of the interaction between E2 and nsP1 is unclear.

Thus it can be seen that these studies on the molecular control of virulence in adult mice have given seemingly different results. This no doubt results from the fact that two sets of studies have used strains that were selected for virulence by passage in infected mouse brain to differing extents, whereas the other two have used naturally occurring virulent and avirulent strains.

**Mechanism of virulence.** The age-dependent virulence of SINV is due to the induction of apoptosis in neurons (Lewis et al., 1996). Two genes that influence apoptosis, Bcl-2 and Bax, have been studied in relation to neurovirulence. An avirulent strain of SINV did not induce apoptosis in cultured cells expressing Bcl-2, whereas a neurovirulent strain did. This property was related to a single amino acid change in the E2 protein that had previously been shown (Lustig et al., 1988, see above) to control neurovirulence (Glu-70 to Lys, Ubol et al., 1994). Neonatal mice were protected from fatal infection and neuronal apoptosis when infected with a recombinant SINV encoding Bax. Thus in these circumstances Bax was neuroprotective (Lewis et al., 1999). Virulent virus expressing Bax and Bcl-2 has also been constructed from a virus vector that is virulent for weanling mice. Animals infected with this virus survived whereas those infected with control wild-type virus succumbed. Thus Bcl-2 and Bax both protect neurons that mediate host...
survival. However, this effect was partial since neither cellular factor could totally suppress the development of hind limb paralysis or the degeneration of neurons in the spinal cord. This indicates that other factors may also mediate survival after infection with virulent virus (Kerr et al., 2002). Schultz et al. (2015) have shown that differentiation of uninfected rat AP-7 neurons was associated with changes in expression of antiviral genes. Expression of key transcription factors was increased, including interferon regulatory factors 3 and 7 (IRF-3 and IRF-7) and Stat-1, suggesting that neuronal maturation may enhance the capacity for antiviral signalling upon infection. However, silencing of IRF expression did not improve virus multiplication in differentiated neurons. Therefore, neuronal differentiation is associated with up-regulation of transcription factors that activate antiviral signalling, but this does not account for maturation-dependent restriction of virus replication.

The role of the immune system in controlling neurovirulence in adult mice has been shown in a study by Kulscar et al. (2015). A neurovirulent strain of SINV causes immune-mediated fatal encephalomyelitis in adult C57BL/6 mice but not in BALB/c mice following intranasal (i.n.) infection. To determine immunological mechanisms underlying these differences, immune responses to infection in these two mouse strains were determined. Resistance to fatal disease in BALB/c mice was associated with better antibody responses, more-rapid virus clearance, fewer Th17 cells, and more-potent regulatory T-cell responses than occurred in susceptible C57BL/6 mice. In the absence of interleukin-10, a component of the regulatory immune response, resistant mice became susceptible to lethal disease.

Age-dependent virulence of SINV strains is reflected in infection of neural cell cultures. CSM14.1 rat neuronal cells are differentiated into neurons by temperature shift. During differentiation, such cells cease dividing, develop neuronal morphology, and express neuron-specific cell markers. SINV infection of undifferentiated CSM14.1 cells resulted in high levels of virus replication and cell death. SINV infection of differentiated CSM14.1 cells resulted in the production of less virus and cells survived. In undifferentiated cells, SINV induced a rapid shutdown of cellular protein synthesis and pE2 (the precursor to E2) was efficiently processed. In differentiated cells, SINV-induced shutdown of cellular protein synthesis was transient and pE2 accumulated in cells. Thus the age-dependent restriction of virus replication, which occurs in vivo, is mirrored in this cell culture system (Vernon & Griffin, 2005).

Differentiated CSM14.1 cells, but not undifferentiated cells, responded to treatment with interferon-gamma (IFN-γ) by decreasing SINV replication. IFN-γ treatment sequentially altered the ratio of genomic to subgenomic viral RNA synthesis, promoted recovery of cellular protein synthesis, reduced viral protein synthesis and inhibited viral RNA transcription within 24 h after treatment. Thus differentiated CSM14.1 cells treated with IFN-γ 24 h after infection responded with increased cell viability and clearance of infectious virus (Burdeinick-Kerr & Griffin, 2005). The cellular pathway involved has been elucidated. IFN-γ-treated SINV-infected differentiated CSM14.1 cells, AP-7 olfactory neuronal cells, and primary dorsal root ganglia neurons triggered prolonged Stat-1 Tyr701 phosphorylation, Stat-1 Ser727 phosphorylation and transient Stat-5 phosphorylation. Inhibition of Jak kinase activity with Jak inhibitor I reversed the neuroprotective activity of IFN-γ in differentiated cells. Thus activation of the Jak/Stat pathway is the mechanism for IFN-γ-mediated clearance of SINV infection from mature neurons (Burdeinick-Kerr et al., 2009).

Further information on the Jak/Stat signalling pathway has been obtained for the adult mouse neuroviral strain AR86 and two closely related avirulent strains, Girdwood and TR339. AR86 was found to rapidly and robustly inhibit tyrosine phosphorylation of Stat1 and Stat2 in response to IFN-γ and/or IFN-β. In contrast, Girdwood and TR339 were inefficient inhibitors of Stat1/2 activation. Decreased Stat activation in AR86-infected cells was associated with decreased activation of the IFN receptor-associated tyrosine kinases Tyk2, Jak1 and Jak2. To identify the viral factor involved, cells were infected with AR86/Girdwood chimeric viruses. A single amino acid determinant, which is required for AR86 virulence, was also required for efficient disruption of Stat1 activation, and this determinant fully restored Stat1 inhibition when it was introduced into the avirulent Girdwood background. This indicates that this virulence determinant (nsP1 Thr-538) plays a critical role in down-regulating the response to type I and type II IFNs (Simmons et al., 2010). Later it was also shown that in the absence of a functional type I IFN system, the attenuated mutant is as virulent as wild-type virus. In addition, the IPS-1-dependent sensing pathway, and to a lesser extent, the TRIF-dependent signalling pathway, contribute to this phenotype (Wollish et al., 2013).

The conclusion of these studies is that the ability of SINV to inhibit Jak/Stat and ISP-1 signalling relates to its in vivo virulence potential.

**Semliki Forest virus**

SFV was first isolated from mosquitoes in Uganda in 1942 (Smithburn & Haddow, 1944) and later found to be neurotropic for mice (Smithburn et al., 1946). SFV is known to cause disease in both animals and humans (Mathiot et al., 1990), and a laboratory death has been ascribed to SFV infection. The patient had neurological disease and at postmortem viral meningoencephalomyelitis was observed. SFV was isolated from both the cerebrospinal fluid and the brain. It is known that the scientist involved was working with virus supernatant from BHK cells, but the route of infection is unknown. Seroconversion in laboratory workers is, however, common (Willems et al., 1979).
Strains of SFV. The designations of strains derived from the original isolates of SFV have been made by Bradish et al. (1971). The first SFV isolation was followed by several other isolations. In particular, an avirulent strain designated A7 was isolated from mosquitoes in Mozambique (McIntosh et al., 1961). Most laboratory strains are derived from this strain and the original isolate. The virulent strain L10 is derived from the original isolate. The avirulent A7(74) strain was derived from A7 by further selection for avirulence. The prototype strain is the strain from which the original infectious clone of SFV was derived, and was the subject of several molecular studies. It appears to be derived from the original isolate, but it has lost some of its virulence, possibly due to an indeterminate number of passages in cell culture. The original infectious clone of SFV, constructed from the prototype strain, is designated pSP6-SFV4; the virus produced by transcription of this infectious clone is designated SFV4 (Glasgow et al., 1991).

Ferguson et al. (2015) have analysed a stock of L10 that has been kept preserved for many years and found it to be a mixture of virus producing small and large plaques, which are avirulent and virulent, respectively. In our own work, samples of the A7(74) variant of A7, and L10, were plaque purified three times on BHK cells before seed stocks were prepared. This was passaged in BHK cells to produce a stock that was passaged once more to produce a working stock. These stocks were frozen in aliquots at −70°C and gave plaques of uniform large size.

Virulent strains such as L10 kill laboratory mice when administered peripherally, but avirulent strains such as A7 induce immunity but do not kill the mice. An initial observation was that virulent strains cause neuronal infection and necrosis, whereas this is less marked for avirulent strains. All strains, with the exception of low doses of SFV4, induce a viraemia and cross the blood–brain barrier when administered peripherally, and virulent strains cause lethal encephalomyelitis 5–7 days after infection. However, avirulent strains induce nonlethal demyelinating disease that generally lasts up to 30 days after infection (Atkins et al., 1985, 1999; Fazakerley, 2004), and are lethal for the developing fetus (Atkins et al., 1995).

Although SFV4 has been designated as virulent, in our hands about 60% of adult mice die when 10⁶ plaque-forming units are given intraperitoneally (i.p.). Ferguson et al. (2015) have reported that at lower doses SFV4 does not cross the blood–brain barrier and so is avirulent. Both SFV4 and L10 kill all adult mice when administered i.n. or i.p., and all strains kill neonatal mice.

Mapping of virulence determinants. Initial attempts to analyse virulence utilized chemically induced attenuated mutants of the virulent L10 strain. Such mutants are partially defective in the efficiency of virus multiplication. Several mutants were isolated and one, designated M9, had lower total RNA synthesis in cell culture than the wild-type strain. When administered peripherally, M9 entered the CNS and induced demyelination rather than lethal encephalitis, and infected mice survived and were immune to challenge with wild-type L10. The isolation of such a mutant probably indicates that the virulent strain has the capacity to induce demyelination, but that this is obscured by death (Barrett et al., 1980; Atkins & Sheahan, 1982; Sheahan et al., 1983).

The avirulent A7 strain is attenuated but multiplies at least as efficiently as the virulent L10 strain in standard cultured cells such as BHK cells (Atkins, 1983; Glasgow et al., 1997). Some studies of virulence utilize i.p. infection, and the A7 (74) strain multiplies at least as efficiently as L10 in peripheral tissue (Fazakerley, 2002). However, it has been found that i.n. infection gives more consistent results and is a more direct route to the CNS. It also obviates the observed variable ability of SFV4 to cross the blood–brain barrier (Santagati et al., 1998; Ferguson et al., 2015). Intranasal infection also targets the olfactory bulb, allowing analysis of early events following CNS infection (Sheahan et al., 1996); this occurs in both mice and rats (Sammin et al., 1999). It is a more sensitive indicator of virulence than i.p. infection, although a higher dose is required to produce initial infection. As is the case for SINV, susceptibility of mice to the avirulent strain of SFV is age-dependent (Oliver et al., 1997).

Analysis of the molecular basis of SFV virulence was facilitated by the construction of an infectious clone of SFV, derived from the prototype strain (Liljestrom et al., 1991). Mutations in the E2 protein of SFV4, including at the E2/E3 cleavage site at position 66, have been described which attenuate the virus when given i.n. or i.p. (Val-168 to Asp, Arg-66 to Leu, Glasgow et al., 1991; Lys-162 to Glu, Glasgow et al., 1994; Val-37 to Ile and Asn-212 to Ser, Santagati et al., 1998). Mutation in the nsP2 nuclear localization signal has also been reported to attenuate the virus (Arg-649 to Asp, Fazakerley et al., 2002). The A7 strain has been sequenced and the sequence compared to the more virulent prototype strain (Santagati et al., 1995; Tarbatt et al., 1997; Santagati et al., 1998; Tuittila et al., 2000). A marked feature of the A7 sequence is the presence of a long nontranslated sequence containing multiple repeats at the 3′ end of the genome. This region is 334 nucleotides longer than in the prototype strain. However, it has no influence on virulence (Santagati et al., 1998). There are multiple mutations throughout the A7 genome compared to the prototype strain, and a large proportion result in amino acid substitutions. It was initially reported that neurovirulence was controlled by the E2 gene (Santagati et al., 1995, 1998). Later the same group reported that neurovirulence was controlled by the nsP3 gene (Tuittila et al., 2000), since it was found that substitution of the nsP3 gene from SFV4 into the A7 strain resulted in lethal neurovirulence. However, within this gene, virulence determination was due to the cumulative effect of pairs of mutations and an opal codon in A7 in place of the Arg 469 codon in SFV4. Also, virulence determinants in the nsP1 and nsP2 genes were found (Tuittila & Hinkkanen, 2003). It was concluded that virulence...
determinants in SFV are distributed over a wide region of the nonstructural genes. Other studies indicated that, although the nsP3 gene was important in the determination of virulence, an accumulation of mutations throughout the genome (Tarbatt et al., 1997), including the 5’ nontranslated region (Logue et al., 2008), was necessary for full virulence. The anomaly between these results may have been due to the use of i.n. infection (which is a more sensitive indicator of neurovirulence) as well as p.i. infection. In addition, a study by Weger-Lucarelli et al. (2016), in which envelope protein chimeras between CHIKV and SFV were analysed, has again shown the importance of the E2 gene in determining the pathogenesis of SFV, since demyelination and neurovirulence were associated primarily with one and to a lesser extent a second, of the three domains of the E2 protein. Deletions in the C-terminal variable region of the nsP3 gene have been shown to attenuate virulence (Galbraith et al., 2006). Saul et al. (2015) have constructed infectious clones of L10 and A7(74) and confirmed that transfer of the nsP3 gene from L10 to A7(74) confers virulence. However, transfer of the nsP3 gene from A7(74) to L10 resulted in virus which retained virulence. The virulence of this recombinant was reduced by substitutions of amino acid residues in the cleavage site between nsP1 and nsP2 (His-534 to Arg) and in the nsP2 protease (Val-1052 to Glu). Pulse-chase experiments showed that A7(74) and avirulent recombinant virus was characterized by increased processing speed of the cleavage between nsP1 and nsP2. Thus the nsP3 gene is an important virulence determinant, but it is not the only virulence determinant.

One group has studied the interferon sensitivity of virulent and avirulent strains of SFV. It was found that an avirulent strain of SFV showed no cytopathic effect compared to a virulent strain in type-1-interferon-treated cells and that the yield of infectious virus was 100-fold less for an avirulent strain than for a virulent strain. It was concluded that the reduced sensitivity of the virulent L10 strain to the action of IFN allows it to overcome the established IFN-induced antiviral state of the cell, thereby increasing its virulence (Deuber & Pavlovic, 2007). Martikainen et al. (2015) have shown that the virulent SFV4 strain is tolerant to the antiviral effect of type I interferon in mouse glioma cells. The IFN tolerance was associated with the viral nsP3–nsP4 gene region and distinct from the genetic loci responsible for SFV neurovirulence. This tolerance was in contrast to the avirulent A7(74) strain which was sensitive to IFN. However, it has also been shown that both virulent and avirulent strains induce a cytopathic effect in fibroblasts from interferon receptor knockout mice (IFNAR −/− MEFs), and that when used to infect IFNAR −/− mice, the mice succumb to infection by both strains (Fragkoudis et al., 2007).

As well as being used as a model for the study of alphavirus pathogenesis, SFV has been developed as a vector for the construction of vaccines and cancer therapy agents. This work will not be described in detail here since it has been reviewed elsewhere (Atkins et al., 2008). However, preliminary experiments have been carried out to develop derivatives of the SFV4 strain for the treatment of brain tumours in mice. In the course of this work, it has been necessary to modify the pathogenicity of the virus to control its cell tropism so that it only infects tumour tissue. In an initial study, a replicative SFV4 carrying six tandem targets for the neuron-specific micro-RNA miRT124 between the viral nsP3 and nsP4 genes was constructed. When administered i.p. to adult BALB/c mice, SFV4-miRT124 showed attenuated spread into the CNS and increased survival. Intracranial infection of adult mice showed reduced infection of neurons in the brain, but led to the infection of oligodendrocytes in the corpus callosum. Thus peripheral replication was not affected, indicating neuron-specific attenuation (Ylösmäki et al., 2013). In a second study, it was shown that in contrast to the attenuated strain A7(74) and its derivatives, SFV4-miRT124 displayed increased oncolytic potency in CT-2A murine astrocytoma cells both in culture and in the animal, and in human glioblastoma cell lines pretreated with IFN (Martikainen et al., 2015).

**Cell tropism and demyelination.** SFV infection of most types of vertebrate cell in culture leads to cytopathic effect, the basis of which is usually apoptosis. Such is the case for BHK cells in culture, and mixed glial cells in culture also undergo apoptosis, due to the infection of oligodendrocytes. However, cultured neurons undergo necrosis rather than apoptosis (Glasgow et al., 1997). This reflects the observed cell tropism and cell death mechanisms of the virus in the animal (Sammin et al., 1999). Also, the virulent SFV4 strain multiplies to higher titre in cultured neurons than the avirulent A7 strain (Glasgow et al., 1997).

There is some controversy regarding the mechanism of apoptosis by SFV and SINV and the action of the anti-apoptotic gene Bcl-2 in cell culture. The Bcl-2 gene is known to block apoptosis induction in postmitotic neurons (Levine et al., 1991). The induction of apoptosis may be blocked by the action of Bcl-2 in avirulent strains of SINV, and converts a lytic to a persistent infection (Levine et al., 1993). Induction of apoptosis by SINV has been shown to depend on virus entry and not on virus replication (Nava et al., 1998; Jan & Griffin, 1999), whereas induction of apoptosis by SFV depends on viral RNA synthesis (Glasgow et al., 1997, 1998). However, it has also been shown that although the replicase genes of SFV initiate apoptosis, the envelope protein genes accelerate this process by activating the endoplasmic reticulum stress response (Barry et al., 2010). For SFV infection of rat RIN cells, it has been claimed that overexpression of the Bcl-2 gene leads to inhibition of apoptosis (Lundstrom et al., 1997). Infection of rat prostatic adenocarcinoma (AT3) cells with virulent or avirulent SFV triggered an apoptotic cell response, but expression of Bcl-2 delayed this response early in infection and initiated a persistent infection in a proportion of cells (Scallan et al., 1997). Recombinant SFV-like particles expressing the enhanced green fluorescent protein (EGFP) gene induced delayed apoptosis in rat AT3-Bcl-2 cells. SFV-mediated expression of a cloned pro-apoptotic Bax gene by
the vector, however, enhanced apoptosis induction in both AT3-Bcl-2 cells and standard BHK-21 cells (Murphy et al., 2001). In contrast, three cell types highly overexpressing functional Bcl-2 displayed caspase-3 activation and underwent apoptosis in response to infection with SFV and SINV. In all three cell types, overexpressed 26 kDa Bcl-2 was cleaved into a 23 kDa protein. Cleavage occurred at target sites for caspses removing the N-terminal BH4 region essential for the death-protective activity of Bcl-2. Pre-incubation of cells with the caspase inhibitor Z-VAD prevented Bcl-2 cleavage and partially restored the protective activity of Bcl-2 against virus-induced apoptosis. Moreover, a Bcl-2 mutant was resistant to proteolytic cleavage and abrogated apoptosis following virus infection. These findings indicate that alphaviruses can trigger a caspase-mediated inactivation of Bcl-2 in order to evade cell death protection (Grandgirard et al., 1998).

Following i.p. infection of BALB/c mice by avirulent SFV, a viraemia is induced of 3–4-day duration before clearance by antibody responses. The virus then crosses the blood–brain barrier and multiplies in the CNS only. The peak of virus multiplication in the CNS is 4–7 days post-infection, after which virus is cleared for avirulent strains in adult mice and the mice survive infection. For virulent strains, however, the virus continues to multiply and death occurs due to a lethal threshold of damage to neurons (Balluz et al., 1993; Fig. 1a, b). For avirulent strains, inflammatory demyelination occurs, and a peak is reached at about 14 days after infection (Fig. 1c), when no infectious virus can be detected in the CNS. By 20–30 days after infection, remyelination is progressing. Neuronal damage is kept to a minimum in adult mice by age-dependent restriction of the virus in neurons (Fazakerley et al., 1993). Although SFV is highly sensitive to type I interferon, the restriction of avirulent virus in neurons is not due to this (Fragkoudis et al., 2007). The immune mechanisms and inflammatory response occurring in infected mice have been reviewed elsewhere (Taylor et al., 2015), and so will not be described in detail here.

It is known that demyelination is mainly T-cell mediated and lesions are much reduced in nude or T-cell depleted mice (Gates et al., 1984; Fazakerley & Webb, 1987; Subak-Sharpe et al., 1993). However, the infection of oligodendrocytes probably triggers immune-mediated demyelination. The M9 mutant and A7 strain show a tropism for oligodendrocytes early in infection (5–7 days; Fig. 1d) in the animal (Atkins & Sheahan, 1982; Sheahan et al., 1983; Fazakerley et al., 2006), and a similar tropism in neural cell culture (Gates et al., 1985; Atkins et al., 1990; Fragkoudis et al., 2009). It has been confirmed using SFV4 virus expressing fluorescently labelled protein that multiplication occurs in neurons and oligodendrocytes, but not in astrocytes, in the mouse CNS (Fragkoudis et al., 2009).

**Venezuelan equine encephalitis virus**

Venezuelan equine encephalitis (VEE) is a disease of humans and equines prevalent in the northern countries of South America and the southern United States, and can be fatal. VEEV causes encephalitis as well as numerous other forms of disease. Although transmitted mainly by mosquito bite, aerosol infection of humans can occur, and in the 1960s attempts to weaponize the virus were made both in the USA and USSR. Numerous laboratory infections have occurred (Weaver et al., 2004; Weaver & Reisen, 2010; Zacks & Paessler, 2010; Derlet, 2014).

**Pathogenesis in mice.** As with SINV and SFV, avirulent strains of VEEV show age-dependent restriction of replication in neurons (Schultz et al., 2015). In adult mice, virulent strains of VEEV cause a biphasic disease following subcutaneous inoculation. In the initial phase, virus replicates primarily in lymphoid tissue, and induces a high-titre viraemia (MacDonald & Johnston, 2000). The virus then invades the CNS from the circulation, and encephalitis ensues (Charles et al., 1995). At the earliest times that the virus is observed in the CNS, it is in areas of the brain involved in olfaction. Virus replication within olfactory and dental tissues induces the opening of the olfactory pathways. Virus later disseminates along connected circuits within the brain, resulting in disseminated meningoencephalitis. Further information was obtained by the use of VEEV replicon particles (VRP). These are particles derived from the VEEV vector that are able to go through one round of multiplication in an infected cell but no further. Replication of VRP in the nasal

![Fig. 1](image-url). Brain lesions in 40–60-day-old BALB/c mice following infection with 10⁶ plaque-forming units of Semliki Forest virus. (a) Neuronal necrosis in the piriform cortex, SFV4, intranasal (i.n.), 4 days post-infection (dpi). Haematoxylin and eosin. Bar, 50 μm. (b) SFV antigen in neurons and neuronal cell processes in the pyriform cortex, SFV4, i.n., 4 dpi. Rabbit anti-SFV polyclonal IgG and haematoxylin counterstain. Bar, 25 μm. (c) Demyelinated plaque in the mid-brain, SFV-M9, intraperitoneal (i.p.), 14 dpi. Toluidine blue stain. Bar, 8 μm. (d) Electron micrograph of virus particles in the cytoplasm of an oligodendrocyte in the mid-brain, SFV-M9, i.p., 5 dpi. Bar, 160 nm.
mucosa induced the opening of the blood–brain barrier, allowing peripherally administered VRP to invade the brain. Peripheral VEEV infection was characterized by a biphasic opening of the blood–brain barrier. This suggests that VEEV initially enters the CNS through the olfactory pathways and initiates viral replication in the brain, which induces the opening of the blood–brain barrier, allowing a second wave of invading virus from the periphery to enter the brain (Schafer et al., 2011). Cell death in the brain is probably due to apoptosis of neurons (Jackson & Rossiter, 1997).

Mapping of virulence determinants. Initially, the nucleotide sequence of the 265 structural region of the virulent Trinidad Donkey strain and the vaccine TC-83 strain were determined. All five of the nucleotide changes which produced non-conservative amino acid substitutions were located in the E2 gene. One nucleotide difference was found in the nontranslated region immediately preceding the 5' end of the 265 mRNA. It was thus concluded that the E2 and nontranslated region mutations were candidates for the molecular determinants of neurovirulence (Johnson et al., 1986).

Further developments were based on the construction of infectious clones (Davis et al., 1989). Attenuated mutants were isolated by selection for rapid penetration of cultured cells. Sequence analysis of these mutants identified candidate attenuating mutations at four loci in the E2 gene: a double mutation at E2 codons 3 and 4 (both Glu to Lys) and three further single substitutions in E2 (Glu-76 to Lys, Thr-120 to Lys, and Glu-209 to Lys). Each candidate mutation was reproduced using site-directed mutagenesis of a full-length cDNA clone. Characterization of these mutants showed that mutation at each of the four loci in the E2 gene was sufficient to confer both the accelerated penetration and attenuation phenotypes (Davis et al., 1991). The importance of the E2 protein in pathogenesis was shown in further studies. A full-length cDNA clone of a virulent strain of VEEV was used as a template for in vitro mutagenesis to produce attenuated single-site mutants. The spread of parent or mutant viruses in the mouse was monitored by infectivity, immunocytochemistry, in situ hybridization and histopathology. Virulent VEEV spread through the lymphatic system, produced viraemia, and replicated in several visceral organs. As virus was being cleared from these sites, it appeared in the brain, beginning in the olfactory tracts. A single-site mutant in the E2 glycoprotein (Glu-76 to Lys) blocked pathogenesis at an early stage and required a reversion mutation to spread beyond the site of inoculation (Davis et al., 1994; Aronson et al., 2000). Other mutations in E2 affected other pathogenic properties involving disease progression (Grieder et al., 1995) and clearance from the blood (Bernard et al., 2000). Also, E2 mutations are involved in equine amplification and virulence, and the emergence of epizootic from enzootic strains (Greene et al., 2005).

One study has implicated type I interferon action in the virulence of VEEV. In a mouse model, cDNA-derived virulent strain V3000 inoculated subcutaneously (s.c.) causes high-titre peripheral replication followed by neuroinvasion and lethal encephalitis. A single nucleotide change in the 5' untranslated region of the V3000 genome (G to A at nucleotide 3) resulted in a virus (V3043) that was avirulent in mice. The mechanism of attenuation of the V3043 mutation was studied in vivo and in vitro. Kinetic studies of virus spread in adult mice following s.c. inoculation showed that V3043 replication was reduced in peripheral organs compared to that of V3000. Titres in serum were also lower, and V3043 was cleared more rapidly from the periphery than V3000. Because clearance of V3043 from serum began 1 to 2 days prior to clearance of V3000, the involvement of type I IFN activity in pathogenesis was examined. In IFN α/β receptor knock-out mice, the course of the wild-type disease was extremely rapid, but the mutant V3043 was as virulent as the wild type. Virus titres in serum, peripheral organs, and brain were similar in V3000- and V3043-infected IFN α/β receptor knock-out mice at all time points up to the death of the animals. Consistent with the in vivo data, the mutant virus exhibited reduced growth in vitro in several cell types except cells that lacked a functional IFN α/β pathway. In cells derived from IFN α/β receptor knock-out mice, the mutant virus showed no growth disadvantage compared to the wild-type virus, suggesting that type I IFN plays a major role in the attenuation of V3043 compared to V3000. There were no differences in the induction of type I IFN between V3000 and V3043, but the mutant virus was more sensitive than V3000 to the antiviral actions of type I IFN in two separate in vitro assays, suggesting that the increased sensitivity to type I IFN plays a major role in the in vivo attenuation of V3043 (White et al., 2001).

Eastern equine encephalitis virus

EEEV is a mosquito-transmitted virus with an uncommon incidence in the eastern USA, the Caribbean and South America. In equines, it is invariably fatal, producing lesions in the CNS and other organs (Del Fierro et al., 2001) and it also infects birds (Williams et al., 2000). In humans, eastern equine encephalitis (EEE) is associated with a high rate of morbidity and mortality (30–70%).

Mapping of virulence determinants. Experimental infection of mice with EEEV is usually carried out s.c. in adults. A viraemia is induced before infection of the CNS. To identify genes associated with virulence, infectious cDNA clones of a virulent and avirulent strain were constructed. Two reciprocal chimeric viruses containing structural and non-structural protein gene regions of these strains were constructed. Virulence 50% lethal dose assays and serial sacrifice experiments demonstrated that both structural and nonstructural proteins are contributors to neurovirulence and viral tissue tropism (Aguilar et al., 2008a). A five amino acid deletion made in the capsid protein at position 55 was found to attenuate the virus for mice by abrogating IFN

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resistance, but did not restrict growth in mosquito cells (Aguilar et al., 2008b). To further clarify mechanisms of attenuation, mouse-attenuated BeAr436087 SA-EEEV, considered a vaccine candidate, was compared with mouse-virulent strain FL93-939. Although attenuated, BeAr436087 initially replicated more efficiently than FL93-939 in lymphoid and other tissues, inducing systemic type I IFN release, whereas FL93-939 induced little type I IFN release. BeAr436087 was more virulent than FL93-939 after i.c. injection, confirming that type I IFN responses determined attenuation, but the viruses were similarly sensitive to type I IFN in vitro. Infection with BeAr436087 protected against FL93-939 disease/death, suggesting that the environment produced by BeAr436087 infection attenuated FL93-939. Thus avoidance of type I IFN induction is a major virulence factor for FL93-939 (Gardner et al., 2009). Inactivation of the major heparan-binding domain in the E2 protein of a neurovirulent strain (Lys-71, 74 and 77 to Ala) does not appear to compromise the replication competence of the virus, but has been shown to decrease brain replication and neurologic disease (Gardner et al., 2011, 2013).

Western equine encephalitis virus

Western equine encephalitis (WEE) is seen primarily in states west of the Mississippi River in the USA and in countries of South America. It is commonly a subclinical infection and symptomatic infections are uncommon. However, the disease can cause serious sequelae in infants and children. Unlike EEE, the overall mortality of WEE is low (approximately 4%) and is associated mostly with infection of the elderly. The annual incidence of the virus varies greatly because of the presence of endemic and epidemic forms.

EEEV resembles WEEV and may have been a genetic predecessor of WEEV. The complete nucleotide sequence for WEEV revealed 11 508 nucleotides with an 84% concordance of protein similarity with EEEV. The virus did in fact probably originate as a recombinant of EEEV and SINV. The capsid and nonstructural genes are derived from EEEV whilst the envelope protein genes are derived from a SIN-like virus (Hahn et al., 1988; Weaver et al., 1993, 1997, Netolitzky et al., 2000).

Analysis of virulence. The virus is given either i.c. or i.n. to mice, and following infection there is a 50% mortality that is not dose-dependent. However, hamsters are fully susceptible to WEEV even when it is given peripherally (Julander et al., 2007). The potential virulence of strains of WEEV in mice has been differentiated. Of three epizootic strains and five enzootic strains, it was found that the enzootic strains were neither neurovirulent nor neuroinvasive, but that the epizootic forms were virulent. Epizootic forms are believed to arise from nonpathogenic strains, which are consistently maintained in enzootic cycles (Bianchi et al., 1993). Variation in infectivity and genetic diversity in the structural proteins may account for differences in virulence in mice. Virulence at the molecular level has been compared among eight strains of WEEV. A lethal i.n. infectivity model of WEEV has been developed in adult BALB/c mice, and all eight strains tested were lethal to adult mice in this model, but they varied considerably in the time to death. Based on the time to death, the strains could be classified into two pathotypes: a high virulence pathotype, consisting of three strains, and a low virulence pathotype, comprising five strains. To analyse genetic diversity in the structural protein genes, 26S RNAs from these eight strains were cloned and sequenced and found to have >96% nucleotide and amino acid identity. A cluster diagram divided the eight strains into two genotypes that matched the pathotype grouping, suggesting that variation in virulence can be attributed to genetic diversity in the structural proteins (Nagata et al., 2006). In a separate study of six North American strains of WEE, a mouse-virulent strain and a mouse-avirulent strain were identified, which diverged most in sequence. The avirulent strain was, however, neuroinvasive (Logue et al., 2009). Like SINV, SFV and VEEV, susceptibility of mice to WEEV is age-dependent for avirulent strains, and dependent on neuronal maturation (Castorena et al., 2008). However, it is interferon-independent and directly mediated by IRF-3 (Peltier et al., 2013).

It has been shown that exchanging the E2 Arg-214 in a mouse-virulent strain of WEEV for the Glu present in an avirulent strain ablated mouse mortality. However, the reciprocal exchange did not confer mouse virulence. Also, the same amino acid change contributed to mosquito infectivity (Mossel et al., 2013).

Chikungunya virus

The remaining alphaviruses of medical importance are Ross River virus, O’nyong-nyong virus and CHIKV. These viruses cause polyarthritis rather than encephalitis as the most common form of disease. Ross River virus and O’nyong-nyong virus do not cause encephalitis and so will not be discussed further. CHIKV, an emerging virus that is endemic in parts of Africa, Asia and the Indian Ocean, and has caused an outbreak in Italy, does infrequently infect the CNS, especially in the young and elderly (Britton et al., 2014; Shaikh et al., 2015; Taraphdar et al., 2015). It is related serologically to SFV (Griffin, 2001). Mouse model systems have been set up to study CHIKV-induced encephalitis (Das et al., 2010; Fig. 2).

Mouse models of CHIKV neuropathogenesis. If neonatal mice are infected i.c. or i.n., most strains of CHIKV multiply in the brain and also infect mouse brain cells in culture. However, for some strains there is age-dependent susceptibility as for other alphaviruses. Astrocytes and neurons are the primary cells infected, which undergo caspase 9-mediated apoptosis (Das et al., 2015). For adult mice, Powers & Logue (2007) reported that CHIKV inoculated i.n. in BALB/c mice led to neuronal infection and tissue necrosis...
in the anterior olfactory lobe. A model reported by Wang et al. (2008) used i.n. infection. Five-week-old C57BL/6 mice developed encephalitis 7 days post-infection with severe multifocal infection and necrosis in the cerebral cortex. Immunohistochemistry techniques revealed that neurons were infected and apoptosis induced while prominent microgliosis and perivascular cuffs were distributed throughout the parenchyma. Moreover, there was neuronal degeneration in the hippocampus and multifocal lymphocytic leptomenigitis. Studies of the neuropathogenesis of CHIKV isolates are in progress, but it has been pointed out that most low-passage field isolates do not produce significant neuropathology in adult mice (Taylor et al., 2015). It has been proposed that macaques may be a better model for studying the neuropathogenesis of wild-type isolates (Inglis et al., 2016).

Although the neuropathology of CHIKV infection of mice has been partially characterized, no studies of the molecular basis of neuropathogenesis have yet been carried out. This should be possible, however, as infectious clones of CHIKV are available and have been used for prototype vaccine construction (Kümmerer et al., 2012; Ahola et al., 2015).

Conclusions

It is clear that the biological mechanism underlying virulence of alphaviruses for the CNS is a lethal threshold of damage to neurons. There is an age-dependent virulence in avirulent strains, and in neonatal mice immature neurons undergo a process of apoptosis. For avirulent strains that enter the CNS, damage to neurons is limited in adult mice. Although IFN regulatory factors are up-regulated in mature neurons, this may not be the only mechanism of resistance. Experiments on SFV have indicated that virulence may be related to the resistance of virulent virus to type I IFN action. It has been found for SINV that resistance to IFN is related to virulence via the Jak/Stat signalling pathway. Further work is required to elucidate the mechanism involved in neuronal susceptibility and resistance for virulent and avirulent strains. Other cell types may, however, be infected; for example, avirulent SFV infects oligodendrocytes and induces immune-mediated demyelination which involves the action of T lymphocytes but allows survival. Infection of oligodendrocytes probably also occurs for virulent strains but its effects are obscured by death. The mechanism by which infection of oligodendrocytes induces immune-mediated demyelination is unknown.

In experiments that have aimed at mapping virulence determinants of alphaviruses at the molecular level, mutations have been found in all genes except the 3’ nontranslated region, the nsP4 gene (the RNA polymerase) and the E3 gene. Most mutations have been reported in the E2 gene, followed by the nsP3 gene. Although the E2 protein may be involved in tropism for neurons and passage across the blood–brain barrier, its role in the induction of apoptosis is unclear. In this regard, the SINV Glu-55 to His mutation has been described as being involved in neurovirulence by two groups (Lustig et al., 1988; Zhu et al., 2015), binding to neural cells (Lee et al., 2002) and apoptosis induction (Ubol et al., 1994). No clear domains of the E2 protein have yet been defined that control virulence for alphaviruses, except perhaps for the heparan-binding domain, which is found in SINV (Ryman et al., 2007) and EEEV (Gardner et al., 2013). Virulence determinants are otherwise found in several different areas of the protein. However, Weger-Lucarelli et al. (2016) have found, by using chimeras constructed from SFV and CHIKV, that one domain of three E2 domains, and another to a lesser extent, are important in determining demyelination and neurovirulence for SFV. Perhaps crystallographic analysis of this protein, as has been carried out for CHIKV (Voss et al., 2010) and SINV (Li et al., 2010), may clarify this in future.

It has been found in separate studies for both SINV (Heise et al., 2003) and SFV (Saul et al., 2015) that naturally occurring avirulent strains carry mutations in the nsP1 protein that accelerate the processing of the nonstructural proteins...
(Arg 534 for SFV, Ile 538 for SINV) compared to virulent strains. Both these mutations are situated within the nsP1–nsP2 cleavage recognition domain.

The nsP3 protein has been shown to be important in the virulence of wild-type strains rather than those produced by neuroadaptation. For both SINV (Suthar et al., 2005) and SFV (Tuitiola & Hinkkanen, 2003), it has been found that mutation of the opal codon within this gene is associated with increased virulence. The opal codon is presumably associated with decreased availability of the RNA polymerase, and this has an opposite effect to the increased processing speed of nonstructural proteins for avirulent virus. The reason for this apparent anomaly is unknown.

The nsP3 protein has a conserved N-terminal and variable C-terminal domain. Deletions made in the variable domain of the SFV4 strain of SFV render the virus avirulent (Galbraith et al., 2006). However, one naturally occurring virulent strain of SINV has an 18-amino acid deletion in the variable domain of nsP3 (Suthar et al., 2005). More information regarding the functions of this protein in infection of neurons may help to further explain the neurovirulence of alphaviruses.

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Alphavirus neuropathogenesis


Alphavirus neuropathogenesis


