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Review Article

The Pathogenesis of Alphaviruses

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Alphaviruses are enveloped single-stranded positive sense RNA viruses of the family Togaviridae. The genus alphavirus contains nine viruses, which are of medical, theoretical, or economic importance, and which will be considered. Sindbis virus (SINV) and Semliki Forest (SFV), although of some medical importance, have largely been studied as models of viral pathogenicity. In mice, SINV and SFV infect neurons in the central nervous system and virulent strains induce lethal encephalitis, whereas avirulent strains of SFV induce demyelination. SFV infects the developing foetus and can be teratogenic. Venezuelan Equine Encephalitis virus, Eastern Equine Encephalitis virus, and Western Equine Encephalitis virus can induce encephalitis in horses and humans. They are prevalent in the Americas and are mosquito transmitted. Ross River virus, Chikungunya virus (CHIKV), and O'nyong-nyong virus (ONNV) are prevalent in Australasia, Africa and Asia, and Africa, respectively. ONNV virus is transmitted by *Anopheles* mosquitoes, while the other alphaviruses are transmitted by culicine mosquitoes. CHIKV has undergone adaptation to a new mosquito host which has increased its host range beyond Africa. Salmonid alphavirus is of economic importance in the farmed salmon and trout industry. It is postulated that future advances in research on alphavirus pathogenicity will come in the field of innate immunity.

1. Introduction

Alphaviruses are small, enveloped, single-stranded positive RNA viruses of the family Togaviridae (Figure 1(a)). Most of them are mosquito transmitted and also infect a variety of mammals and birds. Their intracellular multiplication involves adsorption and receptor-mediated endocytosis into the cell cytoplasm. During intracellular multiplication a gene amplification mechanism results in the formation of a subgenomic RNA species that codes for the structural proteins only and is labelled 26S RNA (Figure 1(b)). The nonstructural and structural proteins are formed from different open reading frames by posttranslational cleavage pathways, and the assembled nucleocapsids bud from areas of the cell membrane or intracellular vesicles that have acquired envelope proteins, thus forming enveloped virions [1].

The genus Alphavirus contains approximately 30 members, which probably diverged a few thousand years ago. Eight alphaviruses, all of which can infect humans as well as other animals, will be discussed here. These are Sindbis virus (SINV), Semliki Forest virus (SFV), Venezuelan equine Encephalitis virus (VEEV), Eastern Equine Encephalitis virus (EEEV), Western Equine Encephalitis virus (WEEV), Ross River virus (RRV), Chikungunya virus (CHIKV), and O'nyong-nyong virus (ONNV). In addition, alphavirus infections of fish have been described, as exemplified by salmonid alphavirus (SAV). Fish viruses are not vector transmitted.

Following infection of mammalian cells in culture, alphaviruses normally induce a cytopathic effect. For SINV and SFV, this has been shown to be the induction of apoptosis. This induction of apoptosis may be blocked by the action of antiapoptotic genes such as Bcl-2 [2]. Induction of apoptosis by SINV has been shown to depend on virus entry and not on virus replication [3, 4], whereas induction of apoptosis by SFV depends on viral RNA synthesis [5, 6]. For mosquito cells, SINV causes persistent virus infection with only partial cytopathic effect [7]. Cell cycle distribution changes following infection with SINV are distinct in mammalian and mosquito cell lines, suggesting that the pathology induced in mosquito cell cultures by SINV infection may be distinct from that which appears in vertebrate cell cultures [8].

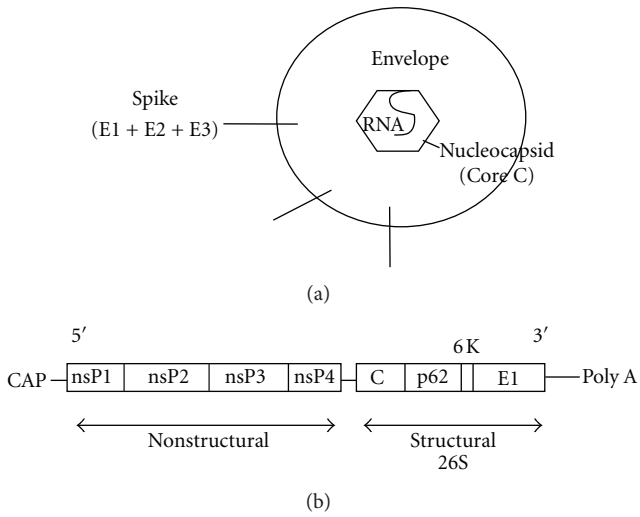

Figure 1: (a) The structure of alphavirus virions (e.g., SFV). (b) Alphavirus genome structure. P62 (also known as PE2) is the precursor to the E2 and E3 proteins.

2. Sindbis Virus

SINV human infection is most common in South and East Africa, Egypt, Israel, Philippines, and parts of Australia. It also causes outbreaks in northern Europe, particularly Finland, and is the causative agent of Ockelbo and Pogosta disease. The symptoms are fever, arthralgia, and rash. Sequence analysis has shown that strains isolated from humans and mosquitoes in northern Europe are closely related. Mosquitoes of *Culex* spp. transmit the virus to humans from avian hosts [9–11].

2.1. Molecular Basis of Virulence. SINV pathogenicity for mice has been the subject of intensive investigation as a model system to understand viral neuropathogenesis, mainly by Griffin and colleagues. Virulence of SINV for mice is age dependent, but a neuaroadapted strain can overcome this age dependence and is virulent for weanling as well as neonatal mice. Other SINV strains are virulent only for neonatal mice. Initially, a variety of strains of SINV of differing pathogenicity for weanling and suckling mice were examined. Hybrid genomes were constructed by replacing restriction fragments in a full-length cDNA clone of SINV, from which infectious RNA can be transcribed, with fragments from cDNA clones of the strains to be examined. Thus the importance of each amino acid difference between the various strains for neurovirulence in weanling and suckling mice was determined. Recombinant viruses containing the nonstructural protein region and the capsid protein region from an avirulent strain and the E1 and E2 glycoprotein regions from a neurovirulent strain were virulent for weanling mice. The control of virulence was narrowed to 3 amino acid changes in the E2 and E1 proteins for weanling mice, but for neonatal mice a gradient of virulence existed involving amino acids in the E1 and E2 proteins [12]. Further data suggested that E2 position 55 plays an important role at early stages of infection of neural cells, including binding [13], thereby facilitating neurovirulence [14]. Recombinant virus studies mapped a determinant of neurovirulence in adult mice to a single amino acid change, glutamine to histidine, at position 55 of the E2 glycoprotein, with histidine conferring neurovirulence. Virus with histidine at position 55 in the E2 protein established infection more efficiently, replicated faster, and achieved higher rates of virus release in neuroblastoma cells than virus with glutamine at position 55. Multiplication of the two viruses in BHK cells was similar. Further data showed that the amino acids at E2 positions 172 and 55 affect both adsorption and penetration of SINV and that these early steps in the replicative pathway contribute to increased neurovirulence [15, 16]. His-55 is important for neurovirulence in older mice and acts by increasing the efficiency of virus replication. In neonatal mice, a separate study has indicated that amino acid 114 in the E2 protein is important for attenuation [17].

In a separate study, SINV was passaged in mouse brain to increase neuroinvasiveness and neurovirulence. Two early passage isolates were neurotropic but did not cause lethal encephalitis. A second pair of isolates, which had undergone more extensive mouse brain passaging, were neurotropic and caused lethal encephalitis. From these isolates, full-length cDNA clones were constructed from which infectious could be transcribed. The strains recovered from these clones were shown to retain the appropriate phenotypes in weanling mice. Construction and analysis of recombinant viruses were used to define the genetic loci determining neuroinvasion. Neuroinvasiveness was determined by loci in the E2 glycoprotein, and in the 5′ noncoding region [18].

Two strains that differ in adult mouse virulence have been used by different authors to define the viral genetic elements that contribute to mouse neurovirulence. The neurovirulent SINV strain AR86 was compared to the closely related but avirulent Girdwood strain. Studies using chimeric viruses demonstrated that genetic elements within the nonstructural and structural coding regions contributed to AR86 neurovirulence. These were a single amino acid change in the E2 protein and 3 changes in the nonstructural region, including an 18-amino acid deletion in nsp3. Replacing these codons in AR86 with those of Girdwood resulted in the attenuation of AR86, while the four corresponding AR86 changes in the Girdwood strain increased virulence to the level of AR86. The attenuating mutations did not adversely affect viral replication in cell culture, and the attenuated viruses established infection in the brain and spinal cord as efficiently as the virulent viruses. However, the virus containing the four virulence determinants grew to higher levels in the spinal cord at late times afterinfection, suggesting that the virus containing the four attenuating determinants either failed to spread or was cleared more efficiently [19].

These three studies on the molecular control of neurovirulence in adult mice have given seemingly different results. However, two sets of studies have used a virus that has been selected for neurovirulence, whereas the other has used naturally occurring virulent and avirulent strains.

2.2. Immunity to Infection. SINV encephalitis in mice has been used as a model system for the study of the development

of immune reactions in the central nervous system (CNS) and clearance of virus from neurons. The immune response is initiated in peripheral lymphoid tissue followed by entry of activated T cells into the cerebrospinal fluid, meninges, and brain parenchyma. Class I and II MHC antigens are expressed extensively on microglia which may present viral antigen produced by infected neurons. Full development of the inflammatory response requires virus-specific T cells, but participating cells include NK cells, gamma delta T cells, monocytes, and B cells. The entry of Ig-secreting B cells corresponds with the appearance of increased amounts of IgG and IgA in the cerebrospinal fluid [20].

Virus clearance from the CNS occurs in three phases: clearance of infectious virus (days 3 to 7), clearance of viral RNA (days 8 to 60), and maintenance of low levels of viral RNA (>day 60). The antiviral immune response is initiated in the cervical lymph nodes with rapid extrafollicular production of plasmablasts-secreting IgM, followed by germinal centre production of IgG-secreting and memory B cells. The earliest inflammatory cells to cross the blood-brain barrier and enter the brain are CD8(+) T cells, followed by CD4(+) T cells and CD19(+) B cells. During the clearance of infectious virus, effector lymphocytes in the CNS are primarily CD8(+) T cells and IgM antibody-secreting cells (ASCs). During clearance of viral RNA, there are more CD4(+) than CD8(+) T cells, and B cells include IgG and IgA ASCs. At later times after infection, ASCs in the CNS are primarily CD19(+) CD38(+) CD138(−) Blimp-1(+) plasmablasts, with few fully differentiated CD38(−) CD138(+) Blimp-1(+) plasma cells. CD19(+) CD38(+) surface Ig(+) memory B cells are also present. The level of antibody to SINV increases in the brain over time, and the proportion of SINV-specific ASCs increases from 15% of total ASCs at day 14 to 90% at 4 to 6 months, suggesting specific retention in the CNS during viral RNA persistence [21].

Viral clearance from the CNS has also been studied using immunodeficient mice. SINV infection and clearance in the CNS of severe combined immunodeficiency (SCID) and C57BL/6 (wild-type) mice, and mice deficient in beta interferon (IFN-β) (BKO), antibody (μMT), IFN-γ (GKO), IFN-γ receptor (GRKO), and both antibody and IFN-γ (μMT/GKO) were studied. Wild-type mice cleared infectious virus by day 8, as did BKO mice, while SCID mice showed persistent virus replication. However, at 3 days after infection, BKO mice had higher titres than wild-type mice. GKO and GRKO mice cleared infectious virus by days 8 to 10 and, like wild-type mice, displayed transient reactivation at 12 to 22 days. μMT mice did not clear virus from the brain and eighty-one days after infection; μMT/GKO mice had not cleared virus, but titres were lower than for SCID mice. This showed that IFN-β is important for early control of CNS virus replication, that antiviral antibody is important for clearance from the brain, and that both antibody and IFN-γ contribute to prevention of reactivation after initial clearance [22]. Antibody knockout mice have also been infected with SINV. Virus was cleared from spinal cord and brain stem neurons, but not from cortical neurons, and required both CD4 and CD8 T cells. Infection with cytokine-expressing recombinant viruses, and mice lacking functional CD8 T cells, suggested that T cells used interferon-gamma in clearing virus [23, 24].

The inflammatory response to infection can also contribute to virulence. This has been shown using 7-acetyl-5-(4-aminophenyl)-8(R)-methyl-8,9-dihydro-7H-1,3-dioxolo-(4,5-h)-benzodiazepine (talampanel), a member of the 2,3 benzodiazepine class of noncompetitive AMPA glutamate receptor antagonists. Talampanel-treated mice were protected from neurovirulent SINV-induced paralysis and death. Examination of the brain during infection showed significantly less mononuclear cell infiltration and no increase in astrocyte expression of glial fibrillary acidic protein in treated mice compared with untreated mice. Lack of CNS inflammation was due to failure of treated mice to induce activation and proliferation of lymphocytes in secondary lymphoid tissue after infection. Antibody responses were also suppressed by talampanel treatment, and virus clearance was delayed [25]. Also, it has been shown that CD4(+) T cells can promote progressive neuronal death and tissue injury, despite clearance of infectious virus [26].

2.3. Infection of Neurons. Encephalomyelitis is caused by infection of neurons in the brain and spinal cord. Recovery is dependent on immune-mediated control and clearance of virus from these terminally differentiated, essential cells. Immune-mediated clearance of infectious virus from neurons is a noncytolytic process. The major effectors are antibody to the E2 surface glycoprotein produced by B cells, and interferon-gamma produced by T cells. Because infected neurons are not eliminated, viral RNA persists and long-term control is needed to prevent virus reactivation. Virus-specific antibody-secreting cells residing in the nervous system after recovery from infection are likely to be important for long-term control [27].

The age-dependent virulence of SINV strains is reflected in infection of neural cell cultures. CSM14.1 rat neuronal cells can be differentiated into neurons by temperature shift. During differentiation, such cells ceased dividing, developed neuronal morphology, and expressed neuron-specific cell markers. SV infection of undifferentiated CSM14.1 cells resulted in high levels of virus replication and cell death. SV infection of differentiated CSM14.1 cells resulted in the production of less virus and cells survived. In undifferentiated cells, SV induced a rapid shutdown of cellular protein synthesis and pE2 was efficiently processed to E2. In differentiated cells, the SV-induced shutdown of cellular protein synthesis was transient and pE2 (the precursor to E2) accumulated in cells. Thus the age-dependent restriction of virus replication, which occurs in vivo, is mirrored in this cell culture system [28].

Differentiated CSM14.1 cells responded to treatment with 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 24 h after infection with IFN-γ responded with increased cell viability and clearance of infectious virus [29]. The cellular pathway involved has been

elucidated. IFN-γ infected differentiated CSM14.1 cells, AP-7 olfactory neuronal cells, and primary dorsal root ganglia neurons triggered prolonged Stat-1 Tyr_{701} phosphorylation, Stat-1 Ser_{727} 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 [30].

Further information on the Jak/Stat signalling pathway has been obtained by different authors for the adult mouse neurovirulent strain AR86 and two avirulent strains. 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, the closely related SINV strains Girdwood and TR339, which are avirulent in adult mice, were relatively 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 a key virulence determinant plays a critical role in downregulating the response to type I and type II IFNs, which suggests that the ability of alphaviruses to inhibit Jak/Stat signalling relates to their in vivo virulence potential [31].

Death of neurons infected by SINV is usually due to apoptosis [32]. Two genes that influence apoptosis, Bcl-2 and Bax, have been studied in relation to the effect of expression on neurovirulence. It was first shown that an avirulent strain of SINV did not induce apoptosis in cultured cells expressing Bcl-2, but a neurovirulent strain did. The ability to induce apoptosis in Bcl-2 expressing cells was related to a single amino acid change in the E2 protein that controlled neurovirulence [33]. Newborn mice, which are susceptible to fatal infection with SINV virus, were protected from neuronal apoptosis and fatal disease when infected with a recombinant SINV encoding Bax. Thus Bax in these circumstances was neuroprotective [34]. Virulent SFV vectors expressing Bcl-2 and Bax have been constructed from a virus strain that causes hind limb paralysis and death in weanling mice. Weanling mice infected with virulent virus encoding Bcl-2 or Bax survived better than animals infected with control viruses. This finding indicates that Bcl-2 and Bax both protect neurons that mediate host survival. Neither cellular factor, however, could suppress the development of hind limb paralysis or prevent the degeneration of motor neurons in the lumbar spinal cord. This indicates that cell death pathways may differ in different sets of neurons [35].

2.4. Infection of Mosquitoes. As part of its natural transmission cycle, SINV infects mosquitoes as well as vertebrates (avian and human). In both mosquitoes and mosquito cells, SINV initiates a persistent infection. Persistently infected mosquito cells are morphologically indistinct from uninfected cells but display a 30% reduction in growth rate compared to uninfected cells [36, 37]. SINV is able to persistently infect other types of insect cells such as *Drosophila* cells, as well as mosquito cells [37].

The midgut and salivary glands of mosquitoes provide a pathway for virus transmission in nature. Although SINV infection does shorten the mosquito life span, persistent coexistence permits survival of both host and virus [38]. Three studies have shown, by site directed mutagenesis and chimera construction, that infection of midgut epithelial cells, which is required after ingestion of a blood meal containing the virus, is controlled by amino acid changes in the E2 glycoprotein [39–41].

Variations in the activity of RNA interference (RNAi) in mosquitoes could explain, in part, why some mosquitoes are competent vectors of medically important, arthropod-borne viruses (arboviruses) and others are not. Virus-derived small interfering RNAs (viRNAs), 21 nt in length, have been detected in *Aedes aegypti* infected with SINV. viRNAs had an asymmetric distribution that spanned the length of the SINV genome. To determine the role of viRNAs in controlling pathogenic potential, mosquitoes were infected with recombinant alphaviruses expressing suppressors of RNA silencing. Mosquitoes in which the accumulation of viRNAs was suppressed showed decreased survival. These results suggest that an exogenous siRNA pathway is essential to the survival of mosquitoes infected with SINV [42].

2.5. Conclusions. One group has defined the molecular determination of virulence as primarily one amino acid change in the E2 protein, whereas other two groups have defined amino acid changes in the structural and nonstructural regions of the genome. This anomaly is probably due to differences in the strains analysed. The E2 change also determines the ability to induce apoptosis in Bcl-2 expressing cells and in mature neurons. Antibody and IFN-γ contribute to noncytolytic clearance of virus from neurons and infected mouse CNS, via the Jak/Stat pathway. SINV persistently infects mosquitoes and mosquito cells, which is controlled by the E2 protein, and persistent infection is maintained by an antiviral RNAi response.

3. Semliki Forest Virus

Semliki Forest virus was first isolated from mosquitoes in Uganda in 1942 [43] and later found to be neurotropic for mice [44]. It is known to cause disease in both animals and man and is found in central, eastern, and southern Africa. A human outbreak has been described in the Central African Republic among Europeans. The symptoms were fever, severe persistent headache, myalgia, arthralgia, and recovery characterised by asthenia. Eight SFV isolates were obtained from *Aedes aegypti* mosquitoes during this outbreak, as well as isolates from human sera [45]. A laboratory death has been described due to SFV infection, after infection by a virulent strain. The patient had neurological disease and at postmortem examination a typical viral meningoencephalomyelitis was observed. SFV was isolated from both

the cerebrospinal fluid and the brain. It is known that the scientist was working with virus supernatant from BHK cells, but the route of infection is unknown. Seroconversion in laboratory workers is, however, common [46].

3.1. SFV Strains. The first SFV isolation was followed by several other isolations [47]. In particular, an avirulent strain designated A7 was isolated from mosquitoes in Mozambique [48]. Most laboratory strains are derived from this strain and the original isolate, designated L10. The avirulent A7 [49] strain was derived from A7 by further selection for avirulence [47]. The prototype strain, from which the original infectious clone of SFV was derived, appears to be derived from the L10 strain, 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 [50].

Virulent strains such as the L10 strain kill laboratory mice when administered peripherally, but avirulent strains such as A7 and its derivative A7 [49] 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 cross the blood-brain barrier, 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 [51–53] and are lethal for the developing foetus [54].

3.2. Analysis of Virulence. Initial attempts to analyse virulence utilised chemically induced attenuated mutants of the 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, it entered the CNS and induced demyelination rather than lethal encephalitis [55], and infected mice survived and were immune to challenge. 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 [53, 56, 57].

The avirulent A7 strain is attenuated but multiplies at least as efficiently as virulent strains in standard cultured cells such as BHK cells [5, 58]. Initial studies of virulence utilised intraperitoneal (i.p.) infection [59], but it was found subsequently that intranasal (i.n.) infection gives more consistent results and is a more direct route to the CNS. I.n. infection also targets the olfactory bulb, allowing analysis of early events following CNS infection [60]. This occurs in both mice and rats [61]. It is a more sensitive indicator of virulence than i.p. infection, although a higher dose is required to produce initial infection.

Analysis of the molecular basis of SFV virulence was facilitated by the construction of an infectious clone of SFV, derived from the prototype strain [62]. The SFV4 virus, produced by transcription of the plasmid encoding the SFV sequence, kills 60–70% of adult Balb/c mice when given i.p., and all neonatal mice, and 100% of adult mice when given i.n. All adult mice survive when given A7 i.p. or i.n., but neonatal mice die. Mutations in the E2 protein of SFV4 have been described which attenuate the virus when given i.n. or i.p. [50, 63–65].

The A7 strain has been sequenced and the sequence compared to the more virulent prototype strain [63–66]. One of the most marked features of the A7 sequence is the presence of a long untranslated 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 [64]. There are multiple mutations throughout the A7 genome compared to the prototype strain, and a large proportion result in amino acid substitutions. One study indicated that neurovirulence was controlled by the nsP3 gene [67], although within this gene determination was due to the accumulation of mutations [68]. Other studies indicated that, although the nsP3 gene was important in the determination of virulence, an accumulation of mutations throughout the genome [66], including the $5'$ untranslated region [69], was necessary for full virulence, but that deletions in the nsP3 gene attenuated virulence [70]. The nsP3 gene is therefore an important virulence determinant, but it is not the only virulence determinant.

One group has studied the interferon susceptibility 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 I 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 susceptibility 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 [71].

3.3. Demyelination. Following i.p. infection of BALB/c mice by avirulent SFV, a viremia 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 5–7 days after infection, after which virus is cleared for avirulent strains. For virulent strains, however, the virus continues to multiply and death occurs due to a lethal threshold of damage to neurons [72]. Virulent strains such as L10 probably do have the capacity to induce demyelination, as shown by the effect of attenuating mutations, but this is obscured by death. For avirulent strains, the peak of inflammatory demyelination is reached at about 14 days after infection (Figure 2), when no infectious virus can be detected in the CNS, and 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 [73].

Demyelination by avirulent SFV is immune-mediated, since it is much reduced in athymic (nude) mice [49, 74]. Also, depletion of CD8+, but not CD4+, T cells abrogates demyelination [75]. The role of antibody is, however, more controversial. Using antibody-deficient mice, one group has reported that antibody is required for viral clearance from

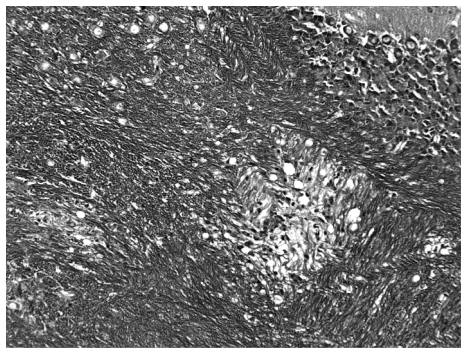

Figure 2: Demyelinated plaque in the white matter of the cerebellum of a BALB/c mouse, 14 days following i.p. infection with an avirulent strain of SFV. Luxol fast blue, ×200. Courtesy of Professor B. J. Sheahan, Veterinary Sciences Centre, UCD School of Agriculture, Food Science and Veterinary Medicine, University College Dublin, Ireland.

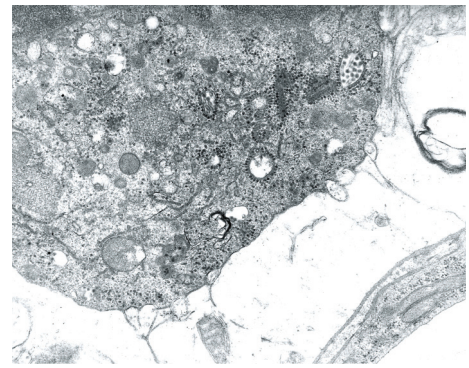

Figure 3: Virus particles and virus nucleocapsids in the cytoplasm of an oligodendrocyte in the midbrain of a BALB/c mouse, 5 days following i.p. infection with an avirulent strain of SFV. Electron micrograph, ×15,000. Courtesy of Professor B. J. Sheahan, Veterinary Sciences Centre, UCD School of Agriculture, Food Science and Veterinary Medicine, University College Dublin, Ireland.

the CNS but is not required for demyelination [76]. However, previous data using a different strain of B-cell-deficient mice showed myelin vacuolation in immunocompetent but not in B-cell-deficient mice, suggesting that CNS infiltrating B cells and antimyelin antibodies contribute to myelin injury [77]. A second study [78] showed that from days 14 to 35 after infection antibodies were produced to myelin proteins. Molecular mimicry has also been described between a viral peptide and a myelin oligodendrocyte glycoprotein peptide [79].

Oligodendrocyte infection (Figure 3) is also be involved in demyelination and may be the triggering event for the immune-mediated demyelination [52]. The M9 and A7 strains show a tropism for oligodendrocytes early in infection (5–7 days) in the animal [54, 71], and a similar tropism in neural cell culture [80, 81]. It has been confirmed using virus expressing fluorescently labelled protein, that multiplication occurs in neurons and oligodendrocytes, but not in astrocytes, in the mouse CNS [82]. A scheme showing the pathogenic mechanisms operating in SFV infection is shown in Figure 4.

Most of the studies described so far have been carried out in BALB/c mice. In this strain demyelination is followed by remyelination and does not persist. However, in the SJL mouse strain, following infection with M9-SFV, small plaques of demyelination and occasional small aggregates of mononuclear leukocytes in the leptomeninges persisted for up to 12 months. This was not associated with detectable persistence of infectious virus, viral antigen, or viral RNA in the CNS [83]. Also, M9-SFV infection induces long-term prolonged expression of proinflammatory cytokines (interferon-gamma and tumour necrosis factor-alpha) in the CNS of the majority of SJL (but not BALB/c) mice, which is not associated with persistence of the virus genome [84]. Thus in SJL mice in this system, infection triggers a long-term inflammatory response in the CNS that is not associated with virus persistence.

Little is known regarding the mechanism of remyelination following SFV infection. However, in one study it was shown that SFV infection of $\gamma\delta$ T cell knockout mice resulted in slower remyelination than infection of wild-type mice. Administration of a peptide epitope of the SFV envelope protein, E2 Th peptide$_2$, resulted in enhanced antibody to this peptide and also more rapid remyelination in $\gamma\delta$ T cell knockout mice [85].

3.4. Cell Interactions. 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 [5]. This reflects the observed cell tropism and cell death mechanisms [61] of the virus in the animal. Also, the virulent SFV4 strain multiplies to higher titre in cultured neurons than the avirulent A7 strain [5].

There is some controversy regarding the action of the antiapoptotic gene Bcl-2 in cell culture. For SINV Bcl-2 has been shown to inhibit apoptosis and initiate a persistent rather than a lytic infection [5]. For SFV infection, overexpression of the Bcl-2 gene leads to inhibition of apoptosis [86]. Infection of rat prostatic adenocarcinoma 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 [87]. Recombinant SFV expressing the enhanced green fluorescent protein (EGFP) gene induced delayed apoptosis in rat AT3-Bcl-2 cells. SFV-mediated expression of a cloned proapoptotic Bax gene by the vector, however, enhanced apoptosis induction both in AT3-Bcl-2 cells and standard BHK-21 cells [88]. 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 caspases removing the N-terminal

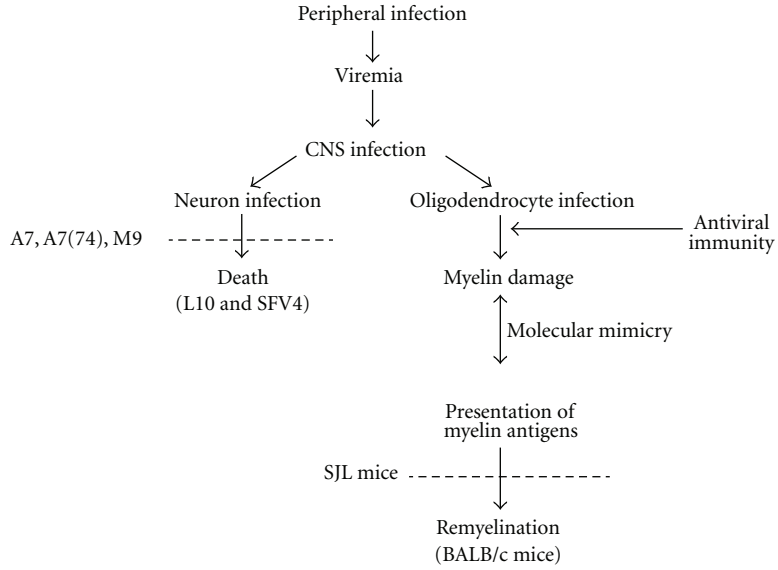

Figure 4: Diagrammatic representation of SFV pathogenesis. The dotted lines represent partial inhibition. Following peripheral (intramuscular, intraperitoneal, or subcutaneous) infection, the virus produces a transient viremia, largely through multiplication in muscle. It then crosses the blood-brain barrier and multiplies in the CNS. All strains of SFV show a tropism for neurons and oligodendrocytes. Infection of neurons with the virulent L10 and SFV4 strains results in a lethal threshold of damage to neurons. Multiplication of the avirulent A7, A7 [49], and M9 strains is partially restricted in neurons and immune intervention occurs to clear the virus before lethal damage can occur. The result of oligodendrocyte infection is myelin damage leading to the presentation of myelin antigens to the immune system and inflammatory demyelination, which could also occur by molecular mimicry. Remyelination occurs in BALB/c mice, but small lesions of demyelination and proinflammatory cytokine secretion occur in SJL mice for up to a year.

BH4 region essential for the death-protective activity of Bcl-2. Preincubation 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 [89].

In mosquito cells, SFV typically establishes a persistent infection but initial SFV infection reduces cellular gene expression. Activation prior to SFV infection of pathways involving Stat/IMD, but not Toll signalling, reduced subsequent virus gene expression and RNA levels. These pathways are therefore able to mediate protective responses against SFV. However, SFV infection of mosquito cells did not result in activation of any of these pathways and suppressed their subsequent activation by other stimuli [90]. SFV cannot prevent the establishment of an antiviral RNAi response or prevent the spread of protective antiviral double-stranded RNA/small interfering RNA (siRNA) from cell to cell, which can inhibit the replication of incoming virus. These results indicate that the spread of the RNAi signal contributes to limiting virus dissemination [91]. The production of virus-derived small interfering RNAs (viRNAs) from viral double-stranded RNA (dsRNA) is a key event in the mosquito cell host response. Deep sequencing of viRNAs and bioinformatic analysis of RNA secondary structures to gain insights into the characteristics and origins of viRNAs showed an asymmetric distribution of SFV-derived viRNAs with notable areas of high-level viRNA production (hot spots) and no or a low frequency of viRNA production (cold spots) along the length of the viral genome. Hot-spot viRNAs were found to be significantly less efficient at mediating antiviral RNAi than cold-spot viRNAs, pointing toward a nucleic acid-based viral decoy mechanism to evade the RNAi response [92].

3.5. Foetal Infection. Different strains and mutants of alphaviruses differ in their effect on developing mouse foetuses when given peripherally to the mother. A7 produced 100% lethality of mouse embryos but was avirulent for their mothers. The neurovirulence mutant M103 did not kill embryos or their mothers but did induce postnatal immunity. This immunity could be induced in utero or by suckling to an immune mother [93]. At gestation day 11, avirulent SFV replicated to high titre in all placentas and was able to persist in the presence of specific maternal antiviral antibodies. There was a delay of at least 1 to 2 days between the initial detection of virus in the placenta and the onset of foetal infection, which resulted in abortion [94]. The state of pregnancy was associated with a marked enhancement of both proliferative and cytotoxic T cell antiviral immune responses after infection of mice with avirulent SFV [95].

Cultured early mouse embryos at the four- to eight-cell stage or the blastocyst stage have been shown to be infected with the A7 strain of SFV after the removal of the zona pellucida, either by pronase treatment or following hatching of blastocysts. The zona pellucida therefore acts as a barrier. Rapid virus production and eventual cytolysis resulted from infection at either stage. For four- to eight-cell embryos the

cytopathic effect was delayed and a proportion of embryos developed to the blastocyst stage [96].

Four temperature sensitive mutants of the avirulent but abortigenic A7 strain of SFV were isolated and their effect on developing foetuses examined. Ts22 and ts14 induced a range of development defects, including developmental arrest, mummification, abortion, and postnatal death. Most surviving offspring were immune. It was concluded that infections of pregnant mice with ts14 and ts22 in particular are good models for analysis of the mechanism of virus-induced developmental defects [97].

The teratogenic ts22 mutant was chosen for further study of teratogenesis. The maximum proportion of skeletal and/or skin defects induced in the 17-day-old foetal mouse occurred following infection of the mother at day 10 of pregnancy. Using immunogold-silver staining, a cDNA probe for an SFV nonstructural sequence, and a riboprobe derived from the same sequence, it was shown that the skin and musculoskeletal systems of foetuses from mothers infected with ts22 were often heavily infected but the CNS was not labelled before day 17 of pregnancy. Mesenchymal cells in the dermis and surrounding developing cartilaginous plates were heavily infected in most foetuses. Other infected foetal tissues contained less viral antigen and nucleic acid; they included the liver, muscle (including myocardium), lung, and kidney. The central nervous system contained only small amounts of viral antigen and nucleic acid. It is proposed that the skeletal and skin defects induced in mouse foetuses by ts22 infection result from the tropism of the virus for mesenchymal cells involved in the development of such tissue [98]. Damage to the neural tube, including open neural tube defects, was detected in foetuses following infection of the mother at days 8 and 10 of pregnancy with both A7 and ts22. For ts22, neural tube damage induced by foetal infection before day 17 of pregnancy appeared to be indirect and caused by virus infection of mesenchymal cells surrounding the developing neural tube [99]. Based on these results, a scheme for the induction of teratogenesis by SFV is shown in Figure 5 [54].

3.6. Conclusions. Like SINV, SFV has been used as a model to analyse the molecular basis of pathogenesis in mice, though with somewhat different results. Unlike standard laboratory strains of SINV, which are given intracerebrally, SFV can be given i.n., i.p., or subcutaneously. If given peripherally, it enters the CNS across the blood-brain barrier. Unlike the situation with SINV, where a small number of mutations determine neurovirulence, and particularly in the E2 protein, SFV virulence determinants appear to be more widely distributed across the virus genome, although the nsP3 gene plays a major role. Both viruses initiate persistent infections in mosquito cells, which are maintained by an antiviral RNAi response. SFV is abortigenic and can be teratogenic.

4. Venezuelan Equine Encephalitis Virus

VEE is a disease of humans and equines prevalent in the northern countries of South America and the southern United States. In humans, VEE is characterised by fever, chills, headache, nausea, vomiting, lumbosacral pain, and myalgia, which may progress to encephalitis. In equines, generalised signs usually appear about 2–5 days after infection with epizootic VEEV, including fever, tachycardia, depression, and anorexia. Most animals go on to develop encephalitis 5–10 days after infection, with signs of circling, ataxia, and hyperexcitability. Death usually occurs about one week after experimental infection. Encephalitis and death are correlative with the magnitude of equine viremia, but even equine-avirulent enzootic strains produce lethal encephalitis when inoculated intracerebrally. This suggests that virulence is related to the ability of VEEV to replicate extracerebrally and spread to the brain rather than to innate neurovirulence. 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 U.S.S.R. Numerous laboratory infections have occurred [100–103].

The etiological agent of VEE was first isolated in 1938 from the brains of fatal equine cases in Yaracuy State, Venezuela. Between 1938 and 1956, only epizootic VEEV strains, later classified as antigenic subtype IAB, were isolated in northern South America. Then, beginning in the late 1950s, VEE and related VEE serocomplex virus strains were isolated in Central America, South America, Mexico, and Florida from sylvatic and swamp habitats in the absence of equine disease. Although these viruses utilise mosquito vectors in a manner similar to epizootic VEEV, they infect primarily small mammalian hosts and are not associated with equine disease. Humans were shown to become infected with these enzootic VEEV strains, with occasional fatal cases. Later antigenic studies demonstrated that these enzootic viruses, along with the epizootic variants, comprise a serocomplex of related alphaviruses. The VEEV complex comprises 14 subtypes and varieties and includes 7 different virus species [103].

4.1. Pathogenesis in Mice. VEEV causes a biphasic disease following subcutaneous inoculation. In the initial phase, virus replicates primarily in the lymphoid tissues, in dendritic cells [104], and induces a high-titre viremia. The virus then invades the CNS from the circulation, and encephalitis ensues. At the earliest times that VEEV-specific in situ hybridization signal is observed in the CNS, it is in areas of the brain involved in olfaction. Virus circulating in the blood appears to seed specific areas of the peripheral nervous system during the viremic lymphoid phase of the illness. Virus replication within olfactory and dental tissues is followed by spread of virus along neural pathways. Virus later disseminates along fibre tracts and connected circuits within the brain, resulting in disseminated meningoencephalitis. Thus haematogenous seeding of the peripheral nervous system is followed by invasion of the CNS by direct neural spread [105]. 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 mucosa induced the opening of the blood-brain barrier (BBB), allowing peripherally administered VRP

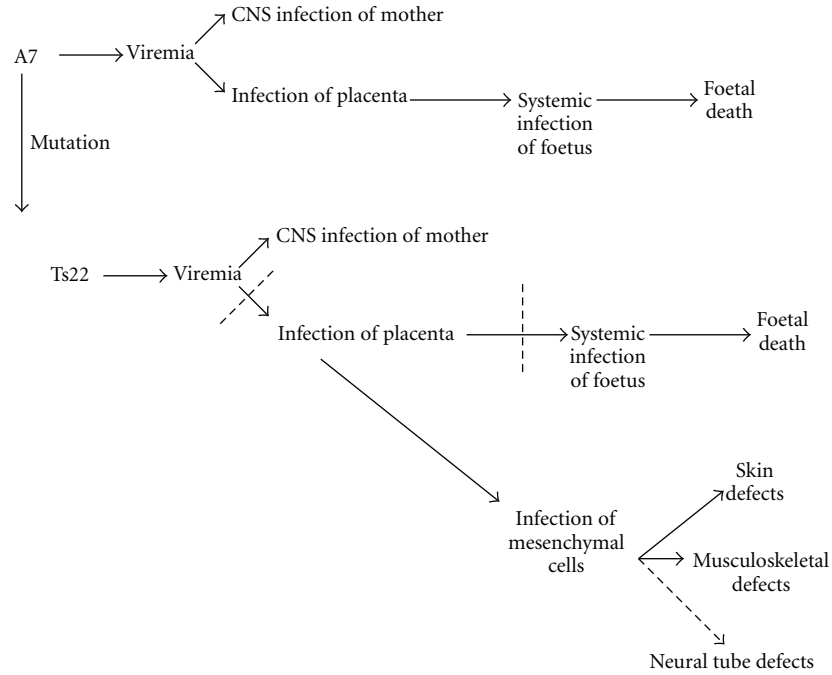

Figure 5: Mechanisms of pathogenicity of the avirulent strain of SFV and the ts22 mutant derived from it for the foetal mouse. Dotted lines represent partial defects in foetal pathogenicity produced by the ts22 mutation. The dotted arrow represents an indirect effect, solid arrows represent direct effects.

to invade the brain. Peripheral VEE virus infection was characterised by a biphasic opening of the BBB. This suggests that VEE initially enters the CNS through the olfactory pathways and initiates viral replication in the brain, which induces the opening of the BBB, allowing a second wave of invading virus from the periphery to enter the brain [106]. Cell death in the brain is probably due to apoptosis of neurons [107].

Gene expression of Toll-like receptors (TLRs) and their associated signalling molecules has been evaluated in the brains of VEEV infected mice. VEEV infection resulted in upregulation of TLRs 1, 2, 3, 7, and 9; chemokines, inflammatory cytokines, interferon (IFN), IFN regulatory factors; and genes involved in signal transduction such as Mcp1, Cxcl10, IL12alpha/beta, IFN-beta, IRF-1, IRF-7, Jun, Fos, MyD88, Nfkb, Cd14, and Cd86. These results demonstrate the upregulation of TLRs and associated signalling genes following VEEV infection of the brain [108]. The type I interferon (IFN) system plays a central role in controlling VEEV infections, and IFN evasion is likely an important determinant of whether these viruses disseminate and cause disease within their hosts. Alphaviruses are thought to limit the induction of type I IFNs and IFN-stimulated genes by shutting off host cell macromolecular synthesis, which in the case of VEEV is partially mediated by the viral capsid protein. Analyses of cells infected with VEEV and VRP demonstrate that viral infection rapidly disrupts tyrosine phosphorylation and nuclear translocation of the transcription factor Stat1 in response to both IFN-beta and IFN-gamma. This effect was independent of host shutoff and expression of viral capsid, suggesting that VEEV uses novel mechanisms to interfere with type I and type II IFN signalling [109].

Using the previously established VRP-mRNP tagging system, a new method has been used to distinguish the host responses in infected cells from those in uninfected bystander cell populations; a rapid innate immune response in the CNS was detected in infected neurons and uninfected bystander cells. Moreover, this innate immune response in the CNS compromised blood-brain barrier integrity, created an inflammatory response, and directed an adaptive immune response characterised by proliferation and activation of microglial cells and infiltration of inflammatory monocytes, in addition to CD4(+) and CD8(+) T lymphocytes. These data suggest that a naive CNS has an intrinsic potential to induce an innate immune response that could be crucial to the outcome of the infection by determining the composition and dynamics of the adaptive immune response [110].

4.2. Molecular Basis of Virulence and Epidemiology. Initially, the nucleotide sequence of the 26S region and deduced amino acid sequence of the virulent Trinidad Donkey strain and the vaccine TC-83 strain were determined. All five of the nucleotide changes which produced nonconservative amino acid substitutions were located in the E2 gene. One nucleotide difference was found in the noncoding region immediately preceding the $5'$-end of the 26S mRNA. It was concluded that the E2 and noncoding region mutations were candidates for the molecular determinants of VEEV neurovirulence [111]. Further developments were based on the construction of infectious clones of VEEV [112]. Attenuated mutants of

VEEV were isolated by selection for rapid penetration of cultured cells. Sequence analysis of these mutants identified candidate attenuating mutations at four loci in the VEEV E2 glycoprotein gene. Each candidate mutation was reproduced in an isogenic recombinant VEEV strain using site-directed mutagenesis of a full-length cDNA clone of VEEV. Characterisation of these molecularly cloned mutant viruses showed that mutation at each of the four loci in the E2 gene was sufficient to confer both the accelerated penetration and attenuation phenotypes [113]. 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 molecularly cloned 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 viremia, 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 blocked pathogenesis at a very early step and required a reversion mutation to spread beyond the site of inoculation [114, 115].

One study has implicated alpha/beta interferon (IFN-alpha/beta) action in the virulence of VEEV. In an adult mouse model, cDNA-derived, virulent V3000 inoculated subcutaneously (s.c.) causes high-titre peripheral replication followed by neuroinvasion and lethal encephalitis. A single change in the $5'$ untranslated region of the V3000 genome resulted in a virus (V3043) that was avirulent in mice. The mechanism of attenuation by 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 also were 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 IFN-alpha/beta activity in VEE pathogenesis was examined. In IFN-alpha/betaR(−/−) 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 the brain were similar in V3000- and V3043-infected IFN-alpha/betaR(−/−) mice at all time points up until the death of the animals. Consistent with the in vivo data, the mutant virus exhibited reduced growth in vitro in several cell types except in cells that lacked a functional IFN-alpha/beta pathway. In cells derived from IFN-alpha/betaR(−/−) mice, the mutant virus showed no growth disadvantage compared to the wild-type virus, suggesting that IFN-alpha/beta plays a major role in the attenuation of V3043 compared to V3000. There were no differences in the induction of IFN-alpha/beta between V3000 and V3043, but the mutant virus was more sensitive than V3000 to the antiviral actions of IFN-alpha/beta in two separate in vitro assays, suggesting that the increased sensitivity to IFN-alpha/beta plays a major role in the in vivo attenuation of V3043 [116].

An analysis of E2 sequences from representative enzootic and epizootic VEEV isolates implicated surface charge changes in the emergence of South American epizootic phenotypes, indicating that E2 mutations are probably important determinants of the equine-virulent phenotype and of VEE emergence. Maximum-likelihood analysis indicated that one change at E2 position 213 has been influenced by positive selection and convergent evolution of the epizootic phenotype [117]. Other evidence has implicated the E2 gene in emergence of epizootic phenotypes. Epizootic subtype IAB and IC VEEV infect the epizootic mosquito vector *Aedes taeniorhynchus*. The inability of enzootic subtype IE viruses to infect this species provides a model for identification of viral determinants of vector infectivity. To map mosquito infection determinants, reciprocal chimeric viruses generated from epizootic subtype IAB and enzootic IE VEEV were tested for mosquito infectivity. Chimeras containing the IAB PE2 envelope glycoprotein E2 precursor gene demonstrated an efficient infection phenotype. Introduction of the PE2 gene from an enzootic subtype ID virus into an epizootic IAB or IC genetic backbone resulted in lower infection rates than those of the epizootic parent. The finding that the E2 envelope glycoprotein, the site of epitopes that define the enzootic and epizootic subtypes, also encodes mosquito infection determinants suggests that selection for efficient infection of epizootic mosquito vectors may mediate emergence [118]. Other genetic studies imply that mutations in the E2 envelope glycoprotein gene are major determinants of adaptation to both equines and mosquito vectors [119, 120]. However, in the guinea pig, the small animal model that best predicts the ability to generate equine viremia, reciprocal chimera construction from closely related epizootic IC, and enzootic ID strains demonstrated that envelope and nonenvelope sequences both contributed to virulence. However, early replication in lymphoid tissues appeared to be primarily envelope dependent [121].

4.3. Conclusions. VEEV is an important mosquito-transmitted pathogen in man and horses which is prevalent in northern South America and central America. Epizootic strains of the virus emerge from enzootic strains largely by selection of E2 protein mutations which enable adaptation to horses and humans.

5. 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, EEE is invariably fatal, producing lesions in the CNS and other organs [122] and it also infects birds [123]. In humans, EEE is associated with a high rate of morbidity and mortality (30–70%). Initial symptoms often progress rapidly to confusion, somnolence, or even coma. Incidence in the United States is roughly 12–17 cases per year. The most recent epidemic occurred in 2003 in North Carolina, where 26 cases were reported. The virus is divided into North (NA) and South American (SA) variants, with 4 major lineages [124]. The prevalence of EEE is increased

in environments with wooded areas adjacent to freshwater swamps and marshes. Most infections occur in summer. The vector population usually dies in winter, and cases of EEE are almost nonexistent in winter months; however, after winter, a repetitive endemic locus of infection may persist.

The enzootic vector for EEEV is the mosquito *Culiseta melanura*, which is responsible for the spring-summer amplification of the virus in the mosquito-bird-mosquito cycle. Occasionally, other mosquito types (e.g., *Coquillettidia perturbans* and *Aedes canadensis*) may act as bridges in horse-to-human transmission [125].

5.1. Animal Models. Three animal models have been used to study EEEV infection. These are mice, hamsters, and marmosets. In mice, most NA and SA strains produce neurologic disease that resembles that associated with human and equine infections. In hamsters, EEE replicates in visceral organs, produces viremia, and penetrates the brain. The pathological manifestations and antigen distribution in the brain of a hamster are similar to those described in human cases of EEEV infection [126]. Marmosets have been tested by using i.n. infection and monitoring for weight loss, fever, anorexia, depression, and neurologic signs. The NA EEEV-infected animals either died or were euthanised on day 4 or 5 after infection due to anorexia and neurologic signs, but the SA EEEV-infected animals remained healthy and survived. Virus was detected in the brain, liver, and muscle of the NA EEEV-infected animals at the time of euthanasia or death. Similar to the brain lesions described for human EEE, the NA EEEV-infected animals developed meningoencephalitis in the cerebral cortex with some perivascular hemorrhages. Thus the marmoset is a useful model of human EEE for testing antiviral drugs and vaccine candidates and has potential for corroborating epidemiological evidence that some, if not all, SA EEEV strains are attenuated for humans [127].

5.2. Interferon Induction and Action. To evaluate the hypothesis that the virulence difference in humans between NA and SA strains is due to a greater ability of NA strains to evade innate immunity, one study compared the replication of NA and SA strains in Vero cells pretreated with interferon (IFN). Human IFN-alpha, -beta, and -gamma generally exhibited less effect on the replication of NA than SA strains, supporting this hypothesis. However, in the murine model, no consistent difference in IFN induction or action was observed between NA and SA strains [128]. In mice, most NA and SA strains produce disease that resembles that associated with human and equine infections. An SA strain that is unable to replicate efficiently in the brain or cause fatal disease in mice yet produces 10-fold higher viremia than virulent EEEV strains has been identified. The avirulent SA strain was also sensitive to human IFN-alpha, -beta, and -gamma, like most SA strains, in contrast to NA strains that were highly resistant. To identify genes associated with IFN sensitivity and virulence, infectious cDNA clones of a virulent NA strain and the avirulent SA strain were constructed. Two reciprocal chimeric viruses containing structural and nonstructural protein gene regions of the NA and SA strains were also constructed. Both chimeras produced fatal disease in mice, similar to that caused by the virulent NA strain. Both chimeric viruses also exhibited intermediate sensitivity to human IFN-alpha, -beta, and -gamma compared to that of the NA and SA strains. Virulence 50% lethal dose assays and serial sacrifice experiments further demonstrated that both structural and nonstructural proteins are contributors to neurovirulence and viral tissue tropism [129]. To further clarify mechanisms of SA-EEEV attenuation, mouse-attenuated BeAr436087 SA-EEEV, considered an EEEV vaccine candidate, was compared with mouse-virulent NA-EEEV strain, FL93-939. Although attenuated, BeAr436087 initially replicated more efficiently than FL93-939 in lymphoid and other tissues, inducing systemic IFN-alpha/beta release, whereas FL93-939 induced little. BeAr436087 was more virulent than FL93-939 in IFN-alpha/beta-deficient mice, confirming that type I IFN responses determined attenuation, but the viruses were similarly sensitive to IFN-alpha/beta 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 IFN-alpha/beta induction is a major virulence factor for FL93-939 [130].

Infectivity of EEE for myeloid lineage cells including dendritic cells and macrophages is reduced compared to that of VEEV, whereas both viruses replicate efficiently in mesenchymal lineage cells such as osteoblasts and fibroblasts. These observations are confirmed in vivo, demonstrating that EEEV is compromised in its ability to replicate within lymphoid tissues, whereas VEEV does so efficiently. The altered tropism of EEEV correlates with an avoidance of serum IFN-alpha/beta induction in vivo, which may allow EEEV to evade the host's innate immune responses and thereby enhance neurovirulence [131].

5.3. Conclusions. EEE is a relatively uncommon disease in eastern USA and northern South America. It is, however, severe in humans and equines in North America, although not in South America. Virulence of EEE appears to be correlated with ability to avoid the IFN-alpha/beta response.

6. Western Equine Encephalitis Virus

In the USA, WEE is seen primarily in states west of the Mississippi River. The disease is also seen in countries of South America. WEE is commonly a subclinical infection; symptomatic infections are uncommon. However, the disease can cause serious sequelae in infants and children. Unlike Eastern Equine Encephalitis, the overall mortality of WEE is low (approximately 4%) and is associated mostly with infection in the elderly. The annual incidence of the virus varies greatly because of the presence of endemic and epidemic forms. The number of cases tends to increase during epidemic years, the worst of which occurred in the western USA and Canadian plains in 1941 and resulted in 300,000 cases of encephalitis in mules and horses and 3336 cases in humans. There have been under 700 confirmed cases in the USA since 1964, with 1-2 cases annually for the past 20 years. However,

this decline in incidence of WEE is not due to a decline in mouse virulence [132].

WEE is spread primarily by the vector mosquito *Culex tarsalis*. *C. tarsalis* is a mosquito that is found on the West Coast of the United States and prefers warm, moist environments. In these locations, cycles of wild bird and mosquito infectivity allow the virus to remain endemic. Thus mosquitoes are the primary vector and birds reservoirs. Epidemic outbreaks in the equine or pheasant population often precede human epidemics. WEE is a summertime infection found in the Western United States, and it is more common in rural areas [133].

Of the alphaviruses, EEEV most closely 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 [134–137].

6.1. Virulence. Mice are usually used as models for WEEV infection, and the virus is given either intracerebrally or intranasally. Following peripheral infection there is a 50% mortality that is not dose dependent. However, hamsters are fully susceptible to WEEV when given peripherally [138].

The potential virulence of strains of WEEV in mice has been differentiated. Of 3 epizootic strains and 5 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 [139]. 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 intranasal infectivity model of WEEV was developed in adult BALB/c mice. All eight strains were 100% lethal to adult mice in this model, but they varied considerably in the time to death. Based on the time to death, the eight strains could be classified into two pathotypes: a high-virulence pathotype, consisting of 3 strains, and a low-virulence pathotype, comprising 5 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 [140]. In a separate study of 6 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 [141].

Like SINV and SFV for mouse and rat cells, WEEV shows a maturation-dependent resistance to cytopathology in human neuronal cells [142].

6.2. Conclusions. WEE is an uncommon mosquito-transmitted infection of humans and equines that has occurred in western USA, South America, and Canada. It is a hybrid virus consisting of sequences derived from EEEV and SINV. Studies of WEEV sequences have been carried out and compared to virulence phenotype, but infectious clones and chimeras for virulence analysis have not yet been developed.

7. Ross River Virus

RRV is an alphavirus transmitted to man by mosquitoes, causing a polyarthritis and arthralgias. It is endemic in Australia where it is the most common mosquito-borne disease affecting humans. Infection can occur as a few sporadic cases, a small outbreak, or a major epidemic.

An RRV outbreak was first described in 1928 in New South Wales, with further outbreaks occurring during World War Two among troops based in the Northern Territory and Queensland. The virus was isolated in 1959 from a mosquito along the Ross River Valley in Queensland. It was confirmed as the cause of RRV disease in 1971 after its isolation from the blood of a boy with the disease.

Approximately 60% of cases arise in tropical and central Queensland (most commonly between January and May) but cases do occur throughout the rest of Australia and outbreaks have occurred in major Australian cities. On average, 5,000 cases are notified each year in Australia, although there is likely to be significant underdiagnosis and underreporting. The largest documented RRV outbreak occurred in the Western Pacific between 1979-1980 and involved more than 60,000 people. Papua New Guinea, Indonesia, and the Solomon Islands continue to have endemic RRV. A similar mosquito-borne alphavirus, the Barmah Forest virus, is the second most common mosquito-borne disease in Australia. The majority of notifications are in Queensland. It causes similar symptoms to RRV.

A variety of species of mosquito act as vectors for the transmission of the virus from animal to human or human to human. The mosquito species most strongly associated with RRV transmission include *Ochlerotatus vigilax*, *Ochlerotatus camptorhynchus*, and *Culex annulirostris*. There is a primary mosquito-mammal cycle involving kangaroos, wallabies, horses, possums, rodents, and other vertebrates. A human-mosquito cycle may occur during epidemic outbreaks.

Progressive resolution of the disease over 3–6 months is usual. Infection in the majority is associated with significant arthralgia and incapacity for at least 3 months after diagnosis. Arthralgias resolve in the majority of patients by 5–7 months. Complete recovery is eventually seen in all cases and infection probably leads to lifelong immunity [143].

7.1. Pathogenesis of Polyarthritis. It has been shown that RRV uses the collagen-binding alpha1beta1 integrin as a cellular receptor [144]. In terms of the E2 protien, which binds to the receptor, both the receptor binding site and neutralization epitopes of RRV are nearby or in the same domain [145]. Virus initially delivered by the mosquito differs from that generated in subsequent rounds of replication in

its ability to infect dendritic cells. Also, unlike mammalian-cell-derived viruses, which induced high levels of type I IFN in infected dendritic cell cultures, mosquito-cell-derived RRV was a poor IFN inducer. This difference may be important with regard to the mosquito-borne virus's ability to successfully make the transition from the arthropod vector to the vertebrate host [146].

Studies on mouse models of polyarthritis have shown the central role of the complement system in the disease. Similar to infection of humans, infection of C57BL/6 mice with RRV results in severe monocytic inflammation of bone, joint, and skeletal muscle tissues. Complement activation products are detected in the inflamed tissues and in the serum of RRV-infected mice. Mice deficient in C3 (C3(−/−)), the central component of the complement system, developed less severe disease signs than wild-type mice. Complement-mediated chemotaxis is essential for many inflammatory arthritides; however, RRV-infected wild-type (WT) and C3(−/−) mice had similar numbers and composition of inflammatory infiltrates within hind limb skeletal muscle tissue. Despite similar inflammatory infiltrates, RRV-infected C3(−/−) mice exhibited less severe destruction of skeletal muscle tissue. Complement activation has also been detected in synovial fluid from RRV-infected patients. Thus complement activation occurs in the tissues of humans and mice infected with RRV and complement plays an essential role in the effector phase, but not the inductive phase, of RRV-induced arthritis [147]. Another study indicated that CR3-dependent signalling at the sites of inflammation contributes to tissue damage and severe disease [148]. The mannose binding lectin (MBL) pathway, but not the classical or alternative complement activation pathways, is essential for the development of RRV-induced disease [149]. Disease in RRV-infected mice was also associated with upregulated macrophage migration inhibitory factor (MIF) expression in serum and tissues, which corresponded to severe inflammation and tissue damage. MIF-deficient (MIF(−/−)) mice developed mild disease accompanied by a reduction in inflammatory infiltrates and muscle destruction in the tissues, despite having viral titres similar to WT mice. In addition, reconstitution of MIF into MIF(−/−) mice exacerbated RRV disease and treatment of mice with MIF antagonist ameliorated disease in WT mice. This suggests that MIF plays a critical role in determining the clinical severity of alphavirus-induced musculoskeletal disease [150]. Using a mouse model of RRV-induced myositis/arthritis, it has been found that myeloid differentiation primary response gene 88 (Myd88)-dependent TLR7 signalling is involved in protection from severe RRV-associated disease. Infections of Myd88- and TLR7-deficient mouse strains with RRV revealed that both Myd88 and TLR7 significantly contributed to protection from RRV-induced mortality, and both mouse strains exhibited more severe tissue damage than WT mice following RRV infection [151]. Thus it appears that RRV-induced arthritis is multifactorial, with several interacting components.

The determinants of arthritic disease have been investigated by chimera construction from infectious clones. An RRV strain (DC5692) has been identified which, in contrast to the T48 strain, does not induce musculoskeletal inflammation in a mouse model of RRV disease. In gain of virulence experiments, substitution of the DC5692 strain nsP1 and pE2 coding regions with those from the T48 strain was sufficient to restore full virulence to the DC5692 strain. These findings indicate that determinants in both nsP1 and pE2 have critical and distinct roles in the pathogenesis of RRV-induced musculoskeletal inflammatory disease in mice [152].

7.2. Conclusions. RRV is a mosquito-transmitted alphavirus causing polyarthritis and arthralgias that normally resolve over several months. It is endemic in Australia and adjacent islands. Infection can occur as sporadic cases, minor outbreaks, or a major epidemic. RRV-induced arthritis has been studied using mouse models. The mannose binding lectin pathway, but not the classical or alternative complement activation pathways, is involved in the development of RRV-induced disease. Disease is also associated with up-regulated macrophage migration inhibitory factor expression in serum and tissues, which corresponds to severe inflammation and tissue damage. Myeloid differentiation primary response gene 88-dependent TLR7 signalling is involved in protection from severe RRV-associated disease. Thus RRV-induced arthritis is multifactorial.

8. Chikungunya Virus

Chikungunya is a mosquito-borne disease first described during an outbreak in southern Tanzania in 1952. The name "chikungunya" derives from the Kimakonde language, meaning "to become contorted," and describes the stooped appearance of sufferers with joint pain. The disease is characterised by abrupt onset of fever accompanied by joint pain. Other common signs include muscle pain, headache, nausea, fatigue, and rash. The joint pain is often very debilitating but usually ends within a few days or weeks. Most patients recover fully but a proportion suffer varying degrees of recurring joint pain.

Both *Aedes aegypti* and *Aedes albopictus* have been implicated in large outbreaks of Chikungunya. Whereas *Ae. aegypti* is confined within the tropics and subtropics, *Ae. albopictus* also occurs in temperate and even cold temperate regions. In recent decades *Ae. albopictus* has spread from Asia to become established in areas of Africa, Europe, and the Americas. Chikungunya occurs in Africa, Asia, and the Indian subcontinent. Human infections in Africa have been at relatively low levels for a number of years, but in 1999-2000 there was a large outbreak in the Democratic Republic of the Congo, and in 2007 there was an outbreak in Gabon. Starting in February 2005, a major outbreak of Chikungunya occurred in islands of the Indian Ocean, including La Réunion, a French territory. A large number of imported cases in Europe were associated with this outbreak, mostly in 2006 when the Indian Ocean epidemic was at its peak. A large outbreak of Chikungunya in India occurred in 2006 and 2007 affecting 1.5 million cases. Several other countries in Southeast Asia were also affected. In 2007 transmission was reported for the

first time in Europe, in a localised outbreak in north-eastern Italy [153].

The biology and pathogenesis of CHIKV has been reviewed recently [154], so here a summary and update will be provided.

8.1. Mosquito Infection. The typical mosquito vector for CHIKV in Africa is *Aedes aegypti*. In causing the Indian Ocean epidemic and other epidemics, the virus was able to adapt to *Aedes albopictus*, through microevolution from its East African origin. This allowed the virus to infect via the human-human transmission cycle and no animal reservoir was involved. This was associated with one mutation in the E1 protein, which allows more efficient infection of *Ae. albopictus* [155]. However, it was later found that E2 mutations are also involved [156], and indeed one E2 mutation enhances the ability of the virus to infect *Ae. albopictus* further but has no effect on fitness in *Ae. aegypti* [157]. Thus the combined effect of the adaptive mutations of the virus to infect *Ae. albopictus* plus an immunologically naive human population that had not previously encountered CHIKV led to the magnitude of the La Réunion outbreak.

8.2. Disease Progression. It has been shown that contrasting immune activation occurs in response to CHIKV infection by mosquito bite or needle inoculation. This may be due to the role of mosquito saliva in these earliest events of CHIKV transmission and infection [158].

Following transmission by mosquito bite, the virus replicates in skin fibroblasts and produces a viraemia, often as high as 10^8 virus particles per mL of blood. Disease onset coincides with rising virus titre, which triggers and innate immune response including the production of type I interferons. The virus disseminates to the liver (endothelial cells), muscle (satellite cells and fibroblasts), joints (fibroblasts), lymphoid tissue (stromal cells, macrophages), and brain (epithelial and endothelial cells). The virus is cleared about one week after infection and then there is evidence of CHIKV-specific adaptive immunity (T-cell- and antibody-mediated responses). About 30% of patients experience long-term sequelae including arthralgia and arthritis [154]. It has been shown that CHIKV infection in humans elicits strong innate immunity involving the production of numerous proinflammatory mediators including high levels of IFNα. Production of IL-4, IL-10, and IFN-γ also suggested development of adaptive immunity, which was confirmed by flow cytometry of circulating T lymphocytes that showed a CD8+ T lymphocyte response in the early stages of the disease [159]. Based on human studies and mouse experimentation, it has been shown that CHIKV does not directly stimulate type I IFN production in immune cells, but infected non-hematopoietic cells sense viral RNA in a Cardif-dependent manner and participate in the control of infection through their production of type I IFNs [160]. However, CHIKV antigens have been detected in the monocytes of infected patients. Using in vitro experimental systems, it has been shown that monocytes could be infected and virus growth could be sustained. CHIKV interactions with monocytes induced a robust and rapid innate immune response with the production of chemokines and cytokines. In particular, high levels of IFN-alpha were produced rapidly after CHIKV incubation with monocytes [161]. In macaques, macrophages have been recognised as the main cellular reservoirs during the late stages of CHIKV infection [162]. Thus the question of the interaction of CHIKV with haematopoietic cells and the induction of innate immune responses is controversial and remains to be fully elucidated.

Interferon-alpha/beta deficiency has been shown to exacerbate arthritogenic disease in mice infected with Chikungunya virus [163]. However, CHIKV replication causes general host shutoff of host macromolecular synthesis, leading to severe cytopathicity in mammalian cells, and inhibits the ability of infected cells to respond to IFN. Alphaviruses may have additional mechanisms to circumvent the host's antiviral IFN response. CHIKV replication is resistant to inhibition by IFN once RNA replication has been established and CHIKV actively suppresses the antiviral IFN response by preventing IFN-induced gene expression. CHIKV infection efficiently blocks Stat1 phosphorylation and/or nuclear translocation in mammalian cells induced by either type I or type II IFN. Expression of individual CHIKV nonstructural proteins showed that nsP2 was a potent inhibitor of IFN-induced JAK-Stat signalling. Thus interferon responses are effectively blocked in mammalian cells by a specific mechanism [164].

8.3. Conclusions. CHIKV has spread from its original domain in Africa to case epidemics in the islands of the Indian Ocean and in East Asia and beyond. In doing so the virus has mutated, in the envelope proteins, to infect a new mosquito vector which has enabled this spread. Innate immunity is involved both in the control of CHIKV infection, the circumvention of host cell immune responses, and in the control of arthritic disease.

9. O'nyong-Nyong Virus

O'nyong-nyong fever is caused by ONNV and causes epidemics in Africa. The name comes from the Nilotic language of Uganda and Sudan and means "weakening of the joints." ONNV was first isolated from human blood and anopheline mosquitoes in Gulu, Uganda, in 1959. There were three major epidemics of O'nyong-nyong fever reported in Africa; in 1959 in East Africa, with more than 2 million cases but no known fatalities; in 1996 in southern Uganda; in 2002 in Central Uganda, near Lake Wamala. Some other outbreaks in Kenya, Tanzania, Malawi, and Mozambique have also been reported.

Clinically the features of ONNV infections include a fever, symmetrical polyarthralgia, lymphadenopathy, generalised papular or maculopapular exanthema, and joint pain. Other symptoms are lymphadenitis, eye pain, and reddening with no discharge, chest pain, and general malaise. No deaths have been reported, and symptomless infections are common.

ONNV virus is unique among mosquito-borne alphaviruses in being primarily transmitted by mosquitoes of

the genus *Anopheles*, for example, *Anopheles gambiae* or *An. funestus* while alphaviruses and flaviviruses are typically transmitted by culicine mosquitoes (*Aedes*, *Culex*, or others). Humans are thought to be the only natural host of ONNV, as other vertebrate reservoirs have not been identified.

ONNV is closely related to CHIKV. Another virus, Igbo Ora, was thought to be a separate virus but closely related to ONNV. However, sequence analysis of this virus indicates that it is a strain of ONNV [165].

9.1. Multiplication in Mosquitoes. Some studies have been made of the multiplication of ONNV in its *Anopheles* mosquito host. While mosquitoes allow ONNV to replicate in them, they also check viral titres so that ONNV infection will result in no harmful effect. Heat shock cognate 70B protein plays a role in suppression of ONNV replication in *A. gambiae* [166]. Therefore, mosquitoes can function as vectors of ONNV transmission to humans while ONNV infection in *A. gambiae* remains asymptomatic. Work has also shown that RNAi is an antagonist of ONNV replication in *A. gambiae*, and allows slow spread to tissues of the mosquito [167].

Chimeric viruses have been constructed from both ONNV and CHIKV, using infectious clones. Virus derived from the infectious clones of ONNV and CHIKV retained the vector specificity of the parental viruses. All six of the chimeras were found to infect *Ae. aegypti* mosquitoes at high rates but only the chimera containing viral genes encoding all of the structural proteins of ONNV was able to infect *A. gambiae* mosquitoes. Thus the structural proteins of ONNV control specificity for its vector host [168]. cDNA microarrays have shown that expression levels of 18 genes were significantly modulated after ONNV infection of mosquitoes, being at least twofold up- or downregulated. Quantitative real-time PCR analysis further substantiated the differential expression of six of these genes in response to ONNV infection. One gene, with sequence similarity to mitochondrial ribosomal protein L7, was downregulated [169].

The alphavirus RNA encodes four different nonstructural proteins, nsP1, nsP2, nsP3, and nsP4. The polyprotein P123 is produced when translation terminates at an opal termination codon between nsP3 and nsP4. The polyprotein P1234 is produced when translational readthrough occurs or when the opal termination codon has been replaced by a sense codon in the alphavirus genome. Evolutionary pressures have maintained genomic sequences encoding both a stop codon (opal) and an open reading frame (arginine) in the O'nyong-nyong virus (ONNV) genome, indicating that both are required. Alternate replication of ONNVs in both vertebrate and invertebrate hosts may determine predominance of a particular codon at this locus in the viral quasispecies. It has been shown that a fitness advantage is provided to ONNV by the presence of an opal codon between nsP3 and nsP4, which is related to mosquito infectivity [170].

9.2. Conclusions. ONNV causes sporadic outbreaks of disease in East Africa, the symptoms being mainly arthralgia and fever. It is unique in utilising *Anopheles* mosquitoes as a vector, a property that is controlled by the structural proteins of the virus. Several mechanisms control viral titres so that ONNV infection will result in no harmful effect to the mosquito.

10. Salmonid Alphavirus

The first alphavirus to be isolated from fish was in 1995 with the isolation of salmon pancreas disease virus from Atlantic salmon in Ireland. Subsequently, the closely related sleeping disease virus was isolated from rainbow trout in France. Norwegian salmonid alphavirus (SAV) has been isolated from marine phase production of Atlantic salmon and rainbow trout in Norway. These three viruses are closely related and are now considered to be three subtypes of SAV. SAVs are recognised as serious pathogens of farmed Atlantic salmon and rainbow trout [171]. They have about 32% sequence homology to other alphaviruses and the same genome structure [172].

10.1. Infection of Fish. For sleeping disease virus, viraemia is detected for 4 weeks after infection, beginning at week 6 and with a peak prevalence at week 7. Clinical signs and mortalities appear at week 8. Total mortality from week 6 onward is 6–47%. Virus neutralising antibodies are first detected at week 9, with seroprevalence increasing to 80% by week 20. Geometric mean antibody titres peak at week 17. Histological lesions are first detected at week 7 (first in pancreas, then in heart, kidney, and skeletal muscle), before increasing in prevalence and severity to peak at weeks 9 and 10. The majority of lesions are resolved by week 15 [173]. It is clear also that with some SAVs symptomless infections can occur [174].

Some work has been carried out on SAV infection of cultured fish cells. SAV-3 infection in a permissive salmon cell line (TO cells) results in IFN-alpha and IFN-stimulated gene mRNA upregulation. Preinfection treatment (4 to 24 h prior to infection) with salmon IFN-alpha induces an antiviral state that inhibits the replication of SAV-3 and protects the cells against virus-induced cytopathic effect. The antiviral state coincides with Mx protein. When IFN-alpha is administered at the time of infection and up to 24 h after infection, virus replication is not inhibited, and cells are not protected against virus-induced cytopathic effect. IFN-gamma has only a minor inhibitory effect on SAV-3 replication. Thus SAV-3 is sensitive to the preinfection antiviral state induced by IFN-alpha, while postinfection antiviral responses or postinfection treatment with IFN-alpha is not able to limit viral replication [175].

A microarray-based study has been performed to evaluate the host transcriptomic response during the early stages of an experimentally induced SAV-1 infection in salmon parr. The greatest number of significantly differentially expressed genes was recorded at 3 days after infection, mainly associated with immune and defence mechanisms, including genes involved in interferon I pathways and major histocompatibility complex class I and II responses. Genes associated with apoptosis and cellular stress were also found to be differentially expressed, as were genes involved in inhibiting

viral attachment and replication. The findings of the study reflected mechanisms used by the host to protect itself during the early stages of SAV-1 infection. In particular, there was evidence of rapid induction of interferon-mediated responses similar to those seen during mammalian alphavirus infections, and also early involvement of an adaptive immune response [176].

10.2. Conclusions. SAV is an important economic pathogen in salmon and trout farms. However, the interaction of SAV with its host and the intracellular multiplication of SAV are only just beginning to be understood, although induction of interferon and adaptive immune responses do occur in the host.

11. General Conclusions

Of the 9 alphaviruses discussed here, 8 are of human medical importance, whilst one is of economic importance to the farmed fish industry. Two viruses, SINV and SFV, have been utilised as models to analyse the molecular and biological basis of viral pathogenesis. All the mammalian viruses are mosquito transmitted; ONNV is transmitted by anopheline mosquitoes (*Anopheles* funestus and *Anopheles gambiae*), whereas the other viruses are transmitted by culicine mosquitoes (*Aedes*, *Culex*; or others). CHIKV has undergone an adaptive mutation to alter its mosquito specificity from *Aedes aegypti* to *Aedes albopictus* and in doing so has increased its host range beyond Africa.

The human diseases caused by the mosquito-borne viruses are of two broad types. In the Americas, VEEV, EEEV, and WEEV cause primarily encephalitis, whereas in Australasia and Africa, RRV, CHIKV, and ONNV cause primarily arthhralgia and arthritis. VEE is the most important pathogen in the Americas, and epizootic strains emerge from enzootic strains by selection of E2 protein mutations which enable adaptation to horses and humans and in this way cause periodic epidemics. Mosquitoes are able to survive virus infection and spread disease through the action of innate immunity, particularly the action of RNAi.

The two model viruses studied, SINV and SFV, infect neurons in the CNS. However, for both viruses, multiplication of avirulent strains is restricted in neurons. Avirulent strains of SFV cause immune-mediated demyelination in the CNS, but this is not the case for SINV. This may be related to the ability to infect oligodendrocytes. For SFV, demyelination is immune-mediated and it has been used as a model for human demyelinating disease such as multiple sclerosis.

For all viruses discussed here, innate immunity may be involved both in disease and in defence against virus infection. Future research may be expected to be concentrated in this area.

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