

Recent advances in Japanese encephalitis

Tom Solomon

Departments of Neurological Science and Medical Microbiology, University of Liverpool, Liverpool, United Kingdom; and WHO Center for Tropical Diseases, University of Texas Medical Branch, Galveston, Texas, USA

Japanese encephalitis (JE), the most important cause of epidemic encephalitis worldwide, is confined to Asia, but its geographical area is spreading. West Nile virus, and other closely related flaviviruses, cause similar disease elsewhere. Recent cryoelectron microscopic studies have characterized the flavivirus envelope protein as a new class of viral fusion protein (class II), and examined its arrangement on the virion surface. Changes in the envelope protein's hinge region, or its putative receptor-binding domain, are associated with changes in neurovirulence in animal models of JE. Clinically, JE causes a wide range of presentations, including a polioliike flaccid paralysis. Seizures and raised intracranial pressure are associated with a poor outcome, and may be potentially treatable. A safe efficacious formalin-inactivated vaccine against JE has been available for many years, but is too expensive for use in most Asian countries. A newer live attenuated vaccine has been used in China, but its use elsewhere has been restricted by regulatory concerns. A chimeric vaccine in which JE structural proteins are inserted into the 17D yellow fever vaccine backbone is one of several vaccines in development. There are no established antiviral treatments against JE. Interferon alpha was the most promising drug in small open trials, but a recent double-blind placebo controlled trial showed that it did not affect the outcome in children with JE. *Journal of NeuroVirology* (2003) 9, 274–283.

Keywords: arthropod-borne virus; CNS infection; flavivirus; vaccine; West Nile virus

Introduction

Although considered by many in the West to be a rare and exotic disease, Japanese encephalitis (JE) is the most important causes of epidemic encephalitis worldwide, causing an estimated 35,000 to 50,000 cases and 10,000 to 15,000 deaths annually (Tsai, 2000). Outbreaks of encephalitis were described in Japan from the 1870s onwards. The virus was first isolated in 1935, and has been recognized across Asia since then (Figure 1) (Solomon, 2000). JE virus (JEV) is a member of the genus *Flavivirus* (family Flaviviridae) that is transmitted naturally in an

enzootic cycle among birds, pigs, and other vertebrate hosts by mosquitoes—especially *Culex tritaeniorhynchus*. Humans become infected when they encroach upon this enzootic cycle. Closely related mosquito-borne flaviviruses in the JE serogroup cause neurological disease elsewhere across the globe, including St Louis encephalitis virus, Murray Valley encephalitis virus, and West Nile virus. The *Flavivirus* genus also includes yellow fever and dengue, which cause hemorrhagic fevers and fever-arthralgia-rash syndromes, and only occasionally central nervous system (CNS) disease (Solomon *et al*, 2000b), the tick-borne encephalitis viruses, and viruses with no known vector that are not important human pathogens (Figure 2). In recent years, the application of molecular biological approaches have helped elucidate the structure of flaviviruses, giving insights into viral pathogenesis and vaccine development. Detailed clinical studies have allowed a better understanding of the pathophysiology, and the first trial of an antiviral drug has been reported.

Address correspondence to Dr. Tom Solomon, Department of Neurological Science, University of Liverpool, Walton Centre for Neurology and Neurosurgery, Liverpool L9 7LJ, United Kingdom. E-mail: tsolomon@liv.ac.uk

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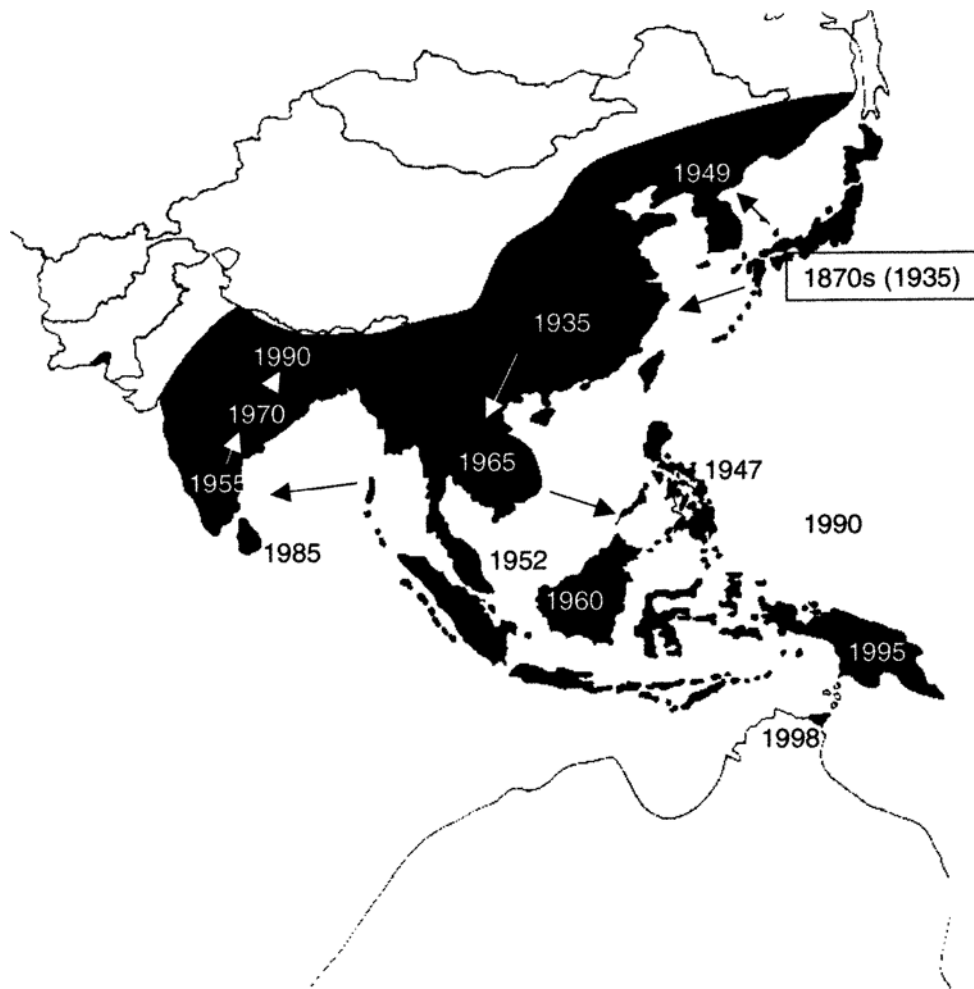


Figure 1 Current distribution of Japanese encephalitis. The approximate dates of the first major outbreaks, or first virus isolations, are shown. Modified from Solomon, 2000, with permission.

Viral replication and morphogenesis

Like the other flaviviruses, the JEV virion consists of a single strand of positive-sense RNA, wrapped in a nucleocapsid and surrounded by a glycoprotein-containing envelope. The RNA comprises a short 5' untranslated region (UTR), a longer 3' UTR, and a between them a single open reading frame (Chambers *et al*, 1990). This codes for a single polyprotein which is co- and post-translational cleaved by viral and host proteases into three structural proteins (core—C; pre-membrane—PrM; and envelope—E), and seven nonstructural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5). The C protein is highly basic and combines with the RNA to form the nucleocapsid. The PrM is closely associated with the E protein, forming a heterodimer, and is thought to act as a 'chaperone' to it, impairing its function until after virion release. Immediately prior to virion release, the PrM protein is cleaved by a furin-like protease to its mature M protein form. This allows the formation

of E protein homodimers, which are thus 'activated' (Stadler *et al*, 1997). The E protein is the largest structural protein, consisting of nearly 500 amino acids with up to two potential glycosylation sites. It is the major target for the humoral immune response, and is thought to be important in viral entry into host cells. Studies with monoclonal antibodies in the late 1980s suggested three antigenic domains (Roehrig *et al*, 1989), and these were confirmed more recently when the three dimensional structure of the ectodomain of the flavivirus tick-borne encephalitis virus was determined by x-ray crystallography (Rey *et al*, 1995). Domain III is the putative receptor binding domain (by which virions attach to the yet-to-be-identified host cell receptor), domain II is the dimerization domain, and domain I has a central μ barrel and is the hinge domain that links the other two domains. Following viral attachment to the cell surface, flaviviruses enter cells by endocytosis, and subsequent fusion of the virus' lipid membrane, with the endosome membrane allows viral RNA to penetrate into

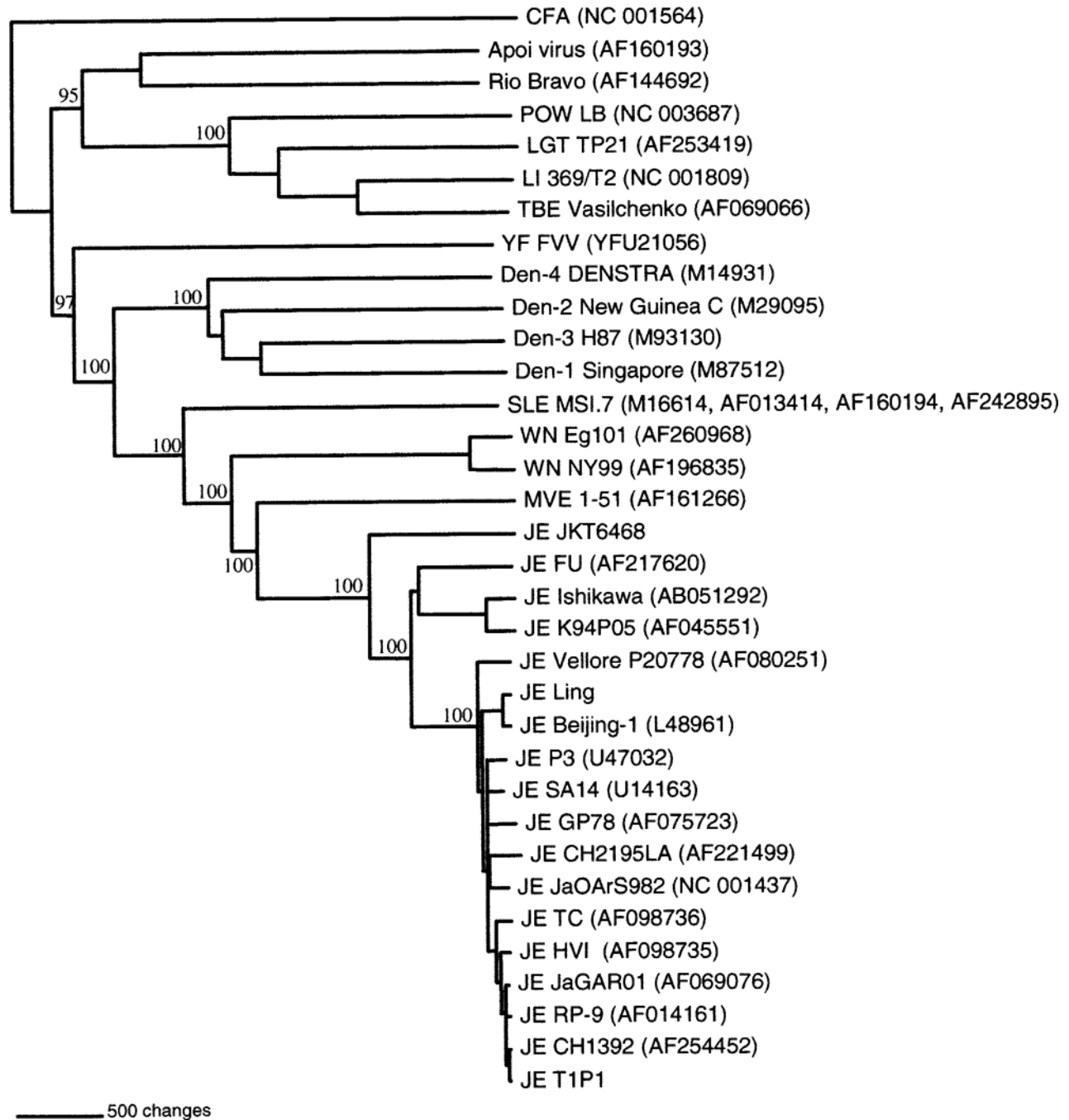


Figure 2 Phylogenetic tree showing the relationship between the flaviviruses. The complete nucleotide sequence of every strain of JEV for which the complete sequence is available, plus representative strains of other important flaviviruses, were aligned using the Vector NTI sequence analysis program, and percentage differences calculated. The phylogenetic tree was then constructed using the maximum likelihood method (PAUP* version 4.04a; Sinauer Associates, Sutherland, MA). The robustness of phylograms was evaluated by 1000 bootstrap replicates. Strain identities are given following the name of the virus, and GenBank accession numbers, or the relevant publications, are shown in parentheses. The tree was outgrouped using cell fusing agent (CFA). Horizontal branch lengths are proportional to the number of changes. POW = Powassan virus; LGT = Langat virus; LI = louping ill virus; TBE = tick-borne encephalitis virus; YF = yellow fever virus; Den = dengue virus; SLE = St Louis encephalitis virus; WN = West Nile virus; MVE = Murray Valley encephalitis virus; JE = Japanese encephalitis virus. From Chiou and Chen, 2001; Jan *et al.*, 1996.

the cytoplasm of the infected cell (Chambers *et al*, 1990). This fusion, which occurs as the pH of the endosome drops, is thought to be mediated by a conformational change around a putative hinge region between domains I and II of the E protein, which brings a μ barrel-shaped fusion peptide at the tip of domain II to insert into the host cell membrane.

Recent cryoelectron microscopy studies of virus-like tick-borne encephalitis virus particles, and of dengue-2 virions, have helped further delineate the structure and organisation of the flavivirus fusion machinery. The structure of recombinant subviral particles (generated by coexpression of tick-borne encephalitis virus PrM and E proteins in mammalian cells) was determined to a resolution of 19 Å, and the atomic structure of the E protein, determined by x-ray crystallography, was fitted into it (Ferlenghi *et al*, 2001). The subviral particle (which is 30 nm across) contained 30 copies of the E dimer arranged in an icosahedral lattice with a triangulation number of 1 ($T = 1$). By extrapolation to a whole flavivirus virion of 50 nm, an arrangement of 90 E dimers in a $T = 3$ lattice was predicted (Ferlenghi *et al*, 2001). And this is what has subsequently been shown for the whole dengue-2 virion. Kuhn and colleagues (2002) determined the structure of the dengue-2 virion with cryoelectron microscopy to 24 Å resolution, and showed the virion has a well-organized outer protein shell, a lipid bilayer, and a less well defined inner nucleocapsid core (Figure 3a and b). Fitting the three-dimensional (3D) structure of the flavivirus E protein showed that the icosahedral scaffold consists of 90 E dimers, lying flat on the surface of the virion. The M protein was thought to be located in a gap between the E dimers (Figure 3c) (Kuhn *et al*, 2002). As the pH-dependent conformational change in the

E proteins occurs, the E homodimers are thought to rearrange into homotrimers, thus exposing a patch of viral membrane for fusion (Kuhn *et al*, 2002). Recent crystallographic studies have shown the E1 protein of alphaviruses bears a striking similarity to the flavivirus E protein, including the arrangement of the three domains, and to the internal fusion peptide (Lescar *et al*, 2001). These flat fusion proteins of flaviviruses and alphaviruses have been designated class II fusion proteins, to distinguish them from the spikelike class I viral fusion proteins of orthomyxoviruses, paramyxoviruses, retroviruses, and filoviruses (Heinz and Allison, 2001). The flavivirus receptor has yet to be identified, but a highly sulphated heparan sulphate molecule may contribute to receptor binding (Chen *et al*, 1997; Su *et al*, 2001).

Clinical epidemiology

In northern temperate regions of Asia, JEV causes large summer epidemics, whereas in southern tropical regions, it causes endemic disease year round (Vaughn and Hoke, 1992). The occurrence of JEV genotypes I and III in northern regions and II and IV in southern regions led to the proposal that different genotypes may explain the differing clinical epidemiology (Chen *et al*, 1990, 1992). However, the recent arrival of a 'northern genotype I' isolate in Australia (Pyke *et al*, 2001), the observation that genotype III is associated with epidemic disease in northern Vietnam and endemic disease in southern Vietnam (Solomon *et al*, 2000a), and the identification of a putative V genotype (Uchil and Satchidanandam, 2001) suggest the current paradigm may need revising.

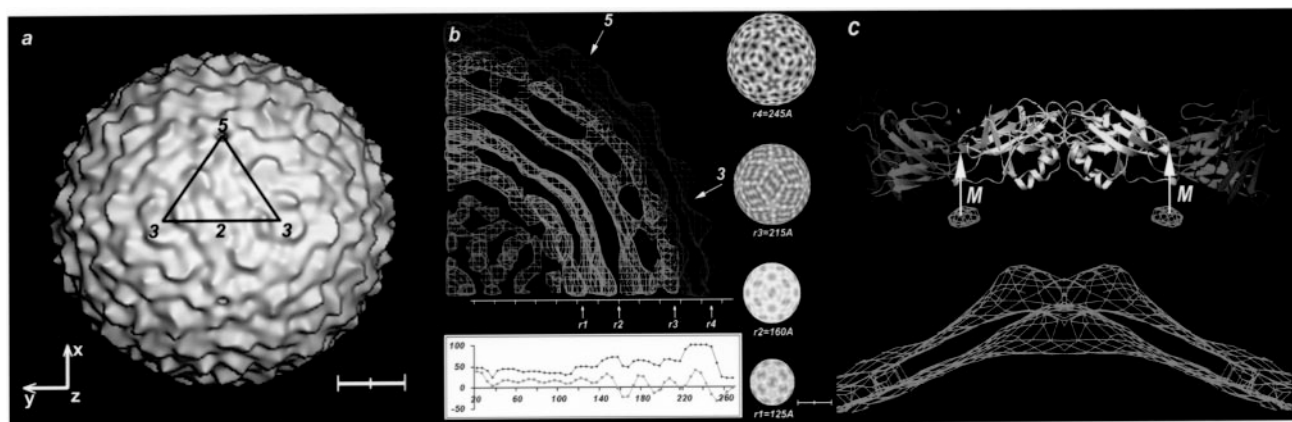


Figure 3 Cryo-electron microscopy of dengue-2 virus, from (Kuhn *et al*, 2002) with permission. (a) Surface shaded representation of the dengue-2 virion cryo-electron microscopy reconstruction at 24 Å resolution, showing the outline of one icosahedral asymmetric unit. The scale bar represents 100 Å. (b) Cross section showing the cryoelectron microscopy density of the dengue-2 virion with a plot of the maximum (upper) and averaged (lower) density. Arrows indicate the position of the 5-fold and 3-fold axes. To the side radial density sections at the defined radii, r1, r2, r3, r4 are shown. Higher density representing protein is shown in dark shading. The scale bar represents 175 Å. (c) Ribbon drawing of the E homodimer. Domains I, II, and III are shown along with the largest uninterrupted electron density peak outside the lipid bilayer (thought to represent the M protein). The white arrows indicate the position of the dimer holes.

Because the rice paddy-breeding *Culex* mosquitoes, which transmit JEV, are unavoidable, the majority of the population in rural Asia has been infected with the virus by early adulthood. Humans become infected during the bite of an infected mosquito. Following inoculation, the virus is thought to replicate in the skin before being transported to local lymph nodes. Langerhans dendritic cells migrating from the skin to the lymph nodes have recently been implicated in this transport in experimental intradermal infection of BALB/C mice with West Nile virus (Johnston *et al*, 2000) and in volunteers receiving candidate live-attenuated dengue virus vaccines (Wu *et al*, 2000). Most JEV infections are asymptomatic, or cause a nonspecific febrile illness. In more severe cases, patients present with a meningoencephalitis, though some present with aseptic meningitis, or a recently described polio-like acute flaccid paralysis (Figure 4) (Solomon *et al*, 1998). This later presentation also occurs in other flavivirus infections, including tick-borne encephalitis virus and West Nile virus (Asnis *et al*, 2000; Kaiser, 1995; Leis *et al*, 2002). Histopathological and imaging studies show viral infection and inflammation in the anterior horn cells of the spinal cord, providing an anatomical correlate for these presentations (Kumar *et al*, 1997; Miyake, 1964). In a similar way, recent radiological studies support earlier pathological studies implicating the basal ganglia, especially the substantia nigra and thalamus in the “parkinsonian” syndromes

seen in JE, which include tremor, cogwheel rigidity, and masklike facies (Kumar *et al*, 1997; Misra and Kalita, 1997; Murgod *et al*, 2001). Other movement disorders and more severe dystonias may also occur (Kalita and Misra, 2000; Solomon and Vaughn, 2002). Seizures are common in JE (Kumar *et al*, 1990; Misra and Kalita, 2001), and it has recently been shown that multiple seizures and status epilepticus were associated with hypoxic brain metabolism, raised intracranial pressure, brainstem herniation syndromes, and a poor prognosis (Solomon *et al*, 2002a). In many patients, the clinical manifestations of status epilepticus were subtle (twitching of a digit, eyebrow, or lip), and could be missed easily, without electroencephalographic monitoring. Rigorous attention to these secondary complications of infection may improve the outcome.

Pathogenesis

In animal models, JEV strains differ in both their neuroinvasiveness (following peripheral inoculation) and neurovirulence (following intracranial inoculation). This may be a consequence of the high viremia achieved by some strains. In mice, JEV strains with higher neurovirulence produce higher viremias than those with lower neurovirulence (Huang and Wong, 1963; Ni and Barrett, 1996). An analysis of the nucleotide and amino acid sequences showed changes



Figure 4 Poliomyelitis-like acute flaccid paralysis in Japanese encephalitis. These two Vietnamese children have a characteristic wasting and weakness of the left quadriceps 1 year after they initially presented with a rapid onset of asymmetrical flaccid paralysis. Reproduced with permission from Solomon *et al*, 1998, and Solomon, 2000.

in the structural, nonstructural, and noncoding regions were associated with the neurovirulent strains. The E protein has been shown to have a major role in determination of virulence phenotype, and single amino acid substitutions are sufficient to cause loss of neurovirulence or neuroinvasiveness (Ni *et al*, 1994, 1995). Two mechanisms mediated by E protein may be involved—attachment of the virus to the receptor and fusion of viral and host cell membranes. The putative receptor binding site of flaviviruses lies in an exposed hydrophilic region of domain III of the envelope protein, which in some mosquito-borne flaviviruses includes the integrin-binding motif arginine-glycine-aspartate (RGD). Substitutions around position E306 on the exposed lateral surface of domain III, at or close to this RGD motif, are associated with loss of neuroinvasiveness (Holzmann *et al*, 1990; Lee and Lobigs, 2000; Ni and Barrett, 1996). Another group of flavivirus variants with altered virulence has amino acid changes in the putative hinge region. For example, several studies of JEV and Murray Valley encephalitis virus neutralization escape variants with low neuroinvasiveness for mice have shown changes around positions 52 and 270–277 of the E protein, both of which lie in this hinge region (Cecilia and Gould, 1991; Hasegawa *et al*, 1992; McMinin *et al*, 1996). A substitution at E279 in a chimeric yellow fever/JEV (see below) was recently shown to affect neurovirulence for mice and monkeys (Monath *et al*, 2002a).

The pathogenesis of flavivirus encephalitis appears to be a combination of direct, virally mediated damage and the host inflammatory response. The host immune response comprises antibody-mediated immunity, particularly against the E and NS1 proteins, and cell-mediated immunity, including cytotoxic T lymphocytes (Johnson *et al*, 1985; Konishi *et al*, 1995). Recent attention has focused on the role of apoptosis in the pathogenesis of arboviral encephalitis. Apoptosis has been shown *in vitro* in a range of cell lines for different flaviviruses, including St Louis encephalitis virus, West Nile virus, Murray Valley encephalitis virus, and JEV (Jan *et al*, 2000; Liao *et al*, 1997; Parquet *et al*, 2002). It has also been shown in animal models of West Nile encephalitis and neurological dengue infection (Despres *et al*, 1998; Xiao *et al*, 2001). *In vitro* apoptosis was associated with a rise in the expression of nuclear factor kappa B (Liao *et al*, 2001), the proapoptotic bax gene (Parquet *et al*, 2002), and reactive oxygen intermediates (Raung *et al*, 2001) including nitric oxide production (Lin *et al*, 2002).

Vaccine development

Safe effective formalin-inactivated vaccines against JEV have been available for at least 30 years (Tsai, 2000). Vaccine produced in Japan from the prototype

Nakayama strain is available internationally under the Biken label, and a vaccine made from Beijing-1 strain is also used in Japan. Although used by travellers, and in rich Asian countries, the Biken vaccine's cost and complex production have meant it has not been used widely in many countries that need it. Moreover, although the risk of serious side effects is actually comparable to other vaccines (about one in one million doses), the vaccine has developed something of a bad reputation (Shlim and Solomon, 2002). These difficulties have stimulated efforts to develop improved vaccines. An inactivated tissue culture-derived vaccine, prepared from JEV P3 strain grown in primary hamster kidney cells, has been used in China since the late 1960s. In the last decade, vero cell-derived inactivated vaccines have also been developed, and are entering clinical trials.

However, live attenuated vaccines appear to offer the best promise for the future, because less virus is needed to produce a satisfactory immune response (making them cheaper to manufacture), and fewer doses are needed (making them easier to give). In the 1980s, the Chinese developed a live attenuated vaccine, named SA14-14-2, by empirically passing JEV strain SA14 through primary hamster kidney cells (Xin *et al*, 1988). Six amino acid changes in the E protein, and three in the nonstructural genes, were associated with the attenuation (Ni *et al*, 1994). SA14-14-2 has proved to be safe, efficacious, and cheap. In open-label studies in China involving more than 600,000 children, the vaccine was shown to have a low incidence of mild, nonspecific side effects (Ma *et al*, 1993). In a retrospective case-control study in which the prevalence of vaccination was compared in 56 JE cases, and nearly 1300 age-matched controls, in affected villages, the vaccine efficacy was shown to be 80% for a single dose and 98% with two doses (Hennessy *et al*, 1996). More than 200 million doses of SA14-14-2 have been delivered in China since 1988, with very few reported side effects. However, regulatory approval for the vaccine's wider use outside of China has been delayed because of concerns about its production. In particular, primary hamster kidney cells have not been used previously for live vaccine production, and there are concerns that the materials used in the production of the original seed viruses may not have complied with international GMP ('good manufacturing practice'). However, given the number of doses administered already to Chinese children without apparent harm, and the current disease burden of JE across Asia, it might be argued that whilst waiting for the development of newer vaccines, the overall benefit of using the current vaccine would outweigh the perceived risk.

One new vaccine in development is a chimeric vaccine in which the PrM and E genes of attenuated JEV strain SA14-14-2 were inserted into an

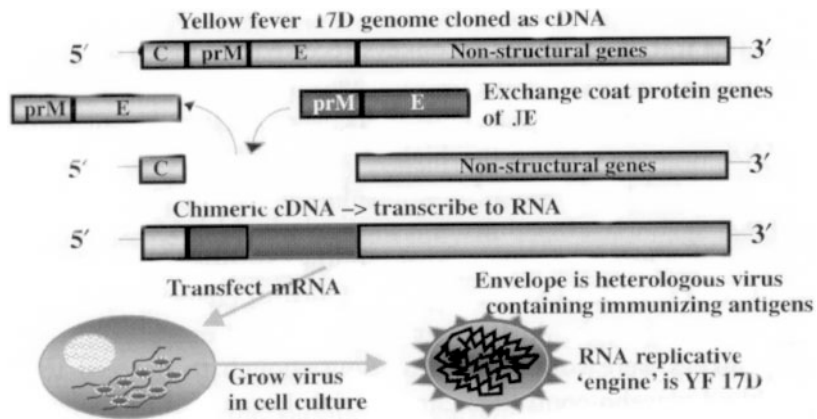


Figure 5 Schematic representation of the Chimeric yellow fever JE vaccine. The PrM and PrE genes of JE strain SA14-14-2 are inserted into the backbone of yellow fever vaccine strain 17D. From Monath, 2002, with permission.

infectious clone of the 17D yellow fever vaccine strain (Figure 5). The chimeric virus (Chimerivax-JE) replicated efficiently *in vitro*, and was shown to be safe in mice and nonhuman primates—being even more attenuated than the original 17D yellow fever strain (Monath *et al*, 2000). Attenuation of the chimeric virus was shown to depend on clusters of at least three of the six amino acid changes in the E protein (Arroyo *et al*, 2001). The vaccine has recently been given to 12 human volunteers in a phase I trial, and was shown to be safe and immunogenic (Monath *et al*, 2002b). A similar approach, using the same 17D yellow fever virus backbone, is being used to develop chimeric vaccines against West Nile and Dengue (Guirakhoo *et al*, 2002). An attenuated vaccinia virus strain has also been used as a vector to deliver JEV structural genes. Although high titres of neutralizing antibodies were elicited in monkeys, and vaccinia-naïve human volunteers, humans who had previously been vaccinated with vaccinia did not produce antibody against JEV (Kanasa-thasan *et al*, 2000), limiting the vaccines potential for future development. Alternative routes of vaccine administration are also being considered in preclinical studies: Ramakrishna and colleagues (1999) showed that oral immunization of mice with live JEV induced a brisk protective immune response against subsequent intracranial challenge.

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Prospects for antiviral treatment

There is no established antiviral treatment for JEV or any other flavivirus infection. A variety of compounds has shown antiviral activity *in vitro* and or animal models of infection (Leyssen *et al*, 2000). Recently, salicylates and nonsteroidal anti-inflammatory drugs were shown to suppress the *in vitro* replication of JEV, and prevent apoptosis of infected cells (Chen *et al*, 2002; Liao *et al*, 2001). This did not appear to be via suppression of nuclear factor kappa B activation, but may be via mitogen-activated kinase (Liao *et al*, 2001). Interferon alpha, a glycoprotein cytokine that is produced naturally in response to viral infections, including JEV (Burke and Morrill, 1987), has been the most promising antiviral candidate. In tissue culture, recombinant interferon is effective against JEV and other arboviruses including West Nile virus (Anderson and Rahal, 2002; Harinasuta *et al*, 1984). In the 1980s, it was given in open trials to a small number of Thai JE patients with encouraging results (Harinasuta *et al*, 1985). However, a recently completed double-blind placebo-controlled trial in Vietnamese children with JE (the first randomized controlled antiviral trial for any flavivirus) showed that although interferon alpha may have delayed the time to death, it made no impact on the overall outcome (Solomon *et al*, 2002b).

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