Japanese encephalitis: development of new candidate vaccines

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Japanese encephalitis (JE) is the most common form of viral encephalitis that appears in the form of frequent epidemics of brain fever throughout Southeast Asia, China and India. The disease is caused by a *Flavivirus* named Japanese encephalitis virus that is spread to humans by mosquitoes. An internationally approved mouse brain-derived inactivated vaccine has been available that is relatively expensive, gives immunity of uncertain duration and is not completely safe. Cell culture-derived inactivated and attenuated JE vaccines are in use in China, but these are not produced as per the norms acceptable in most countries. Several new promising JE vaccine candidates have been developed, some of which are under different stages of clinical evaluation. These new candidate JE vaccines have the potential to generate long-lasting immunity at low cost.

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Since its emergence in Japan in the 1870s, Japanese encephalitis (JE) has become the single most important form of viral encephalitis in the Indian subcontinent and virtually all of Southeast Asia, China and the Asia–Pacific belt. Approximately 3 billion people live in areas endemic to JE where at least 50,000 cases of clinical disease are reported every year. Of these, up to 10,000 cases result in death, mostly among children, and a significant proportion of the survivors live with life-long neurological complications [1]. In recent years, JE has been reported from the hitherto unaffected areas such as northern Australia [2,3] and Pakistan [4]. This, coupled with the high rate of mortality and residual neurological complications in survivors, makes JE a serious public health problem.

Most JE infections are asymptomatic, and only approximately one in 250 infections result in clinical disease. The incubation period of the disease is 4–14 days. Typical JE patients present with nonspecific febrile illness, which may include coryza, diarrhea and rigors. Headache, vomiting and a reduced level of consciousness, often heralded by convulsion, follow this. The principal clinical manifestation of illness is encephalitis. The disease is characterized by seizures, poliomyelitis-like paralysis and Parkinsonian movement disorders. Approximately 60–75% of patients develop encephalitis, with 5–10% showing signs of meningitis. Case fatality rate is 20–30%. A large proportion of the survivors (50–60%) develop neuropsychiatric sequelae that include memory loss, impaired cognition, behavioral disturbances and convulsions [5]. Two epidemiological patterns of JE are recognized. In northern temperate areas, JE occurs in summer epidemics, whereas in southern tropical areas the disease is endemic and occurs all year round. The major epidemiological features of JE are briefly summarized in BOX 1.

The pathogen: Japanese encephalitis virus

JE virus (JEV), the etiological agent for JE first identified in 1924, is an arbovirus belonging to the family Flaviviridae and genus *Flavivirus*. JEV is antigenically linked to other arthropod-borne viruses of the Flaviviridae family, which includes several medically important viruses such as yellow fever (YF), dengue and West Nile encephalitis viruses (TABLE 1). JEV is transmitted to humans by mosquitoes, primarily by *Culex tritaeniorhynchus* that proliferates in close association with animal vertebrate hosts, such as

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swine, water birds (egrets and herons) as well as starlings and chicks. Swine and water birds serve as the primary host for the virus, as these animals develop high levels of viremia upon infection with JEV. Humans and horses develop asymptomatic as well as clinical illness when infected with JEV. As JEV titers in infected humans and horses are insufficient for further transmission by the mosquitoes, these animal species are considered as the dead-end hosts for the virus. Other vertebrate animals that are naturally infected with JEV include donkeys, chicken, ducks, water buffalos, cattle, sheep, mice, bats, snakes and frogs, although their role in further transmission of JEV is not established. The life cycle of JEV in nature is depicted in FIGURE 1.

The single-stranded, positive-sense RNA genome of JEV, consisting of approximately 11 kb [6,7], contains a single open reading frame (ORF) capable of encoding a polyprotein of approximately 3400 amino acids. This polyprotein is subsequently cleaved, co- and post-translationally, by both the host and viral proteases, into three structural; capsid (C) membrane (M) and envelope (E), and seven nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) proteins (FIGURE 2) [8]. A considerable body of information on JEV proteins is extrapolated from data for other flaviviruses due to the fact that a high degree of similarity exists in the genome organization, replication and protein processing of different flaviviruses such as YF and dengue viruses.

The structural component of the virion nucleocapsid is formed by the C protein (12–14 kDa). The premembrane protein (prM), which is 18–19 kDa in size, undergoes cleavage and removal of the N-terminal segment, thereby giving rise to the M protein (8–9 kDa). The cleavage process is presumably linked to the maturation of the E glycoprotein and the development of virus infectivity [9]. Sometimes, the prM protein may become an additional target on virions for neutralizing (Nt) antibodies, due to the incomplete cleavage of the prM protein [10]. The E protein (53–55 kDa) is a typical membrane glycoprotein, consisting of a C-terminal membrane-spanning domain and forms the outer structural protein component of the virus. The E protein, being the major virion antigen, is the principal target for neutralization *in vitro* and *in vivo* by specific antibodies and is responsible for a number of important processes such as virion assembly, receptor binding and membrane fusion [11–13].

Box 1. Epidemiological features of Japanese encephalitis.

- Geographic range:
Indian subcontinent (including Pakistan), south Asia, Southeast Asia, China, Pacific rim, northern Australia
- Major vectors:
Culex tritaeniorrhynchus, *Culex vishnui*, *Culex gelidus*, *Culex fuscocephala*, *Culex pipiens*
- Major vertebrate hosts:
Domestic pigs, migratory/wading birds, such as Asiatic cattle erget, domestic fowl
- High-risk groups:
Children and the elderly in endemic areas and nonimmune adults
- Annual incidence of disease:
30,000–50,000

Table 1. Major human flaviviruses and their endemic areas.

Virus	Endemic areas
Japanese encephalitis	Asia and Asia–Pacific belt
Yellow fever	South America and Africa
Dengue	Tropics, worldwide
West Nile	Europe, Africa, Asia and North America
St Louis encephalitis	North and South America
Murray Valley encephalitis	Australia
Tick-borne encephalitis	Europe and Asia

The NS1 protein (39–41 kDa) is also a glycosylated protein that is believed to be involved in the assembly and release of virions [14], and is found on the cell surface and in the culture medium of infected cells [15]. It has been demonstrated that during the course of infection, this protein evokes a strong antibody response that protects the host against live virus challenge, presumably through a complement-mediated pathway [16]. It has also been argued that the NS1-mediated protection may be due to antibody-dependent cell cytotoxicity [17]. NS2A and NS2B are both relatively small-molecular-weight proteins (22 and 14 kDa, respectively). The former is believed to be involved in the recruitment of RNA templates to the membrane-bound replicase, while the latter might act as a cofactor for the serine protease function of NS3 [18]. The NS3 protein is large (68–70 kDa) and is conserved among flaviviruses. NS3 has several enzymatic activities, such as protease and nucleotide triphosphatase/helicase activities that are implicated in polyprotein processing and RNA replication [19–21]. NS4A and NS4B are relatively small proteins (~16 and 27 kDa, respectively) whose functions are not clear, although they may be involved in the membrane localization of NS3 and NS5 through protein–protein interactions [8], or in the formation of the genomic RNA replication complex [22]. The NS5 protein (~103 kDa) is the largest and most conserved protein among flaviviruses and is considered to be the viral RNA-dependent RNA polymerase (RdRp). It possesses sequence homology to RdRps of other positive-sense RNA viruses, including the GDD motif common to these enzymes [23].

Based on the limited nucleotide sequence data of C/prM and E genes, four genotypes of JEV have been identified [24,25]. The representatives of three of these genotypes have been fully sequenced. Genotype I includes isolates from northern Thailand, Cambodia and Korea. Genotype II includes isolates from southern Thailand, Malaysia, Indonesia and northern Australia. Genotype III includes isolates from mostly temperate regions of Asia,

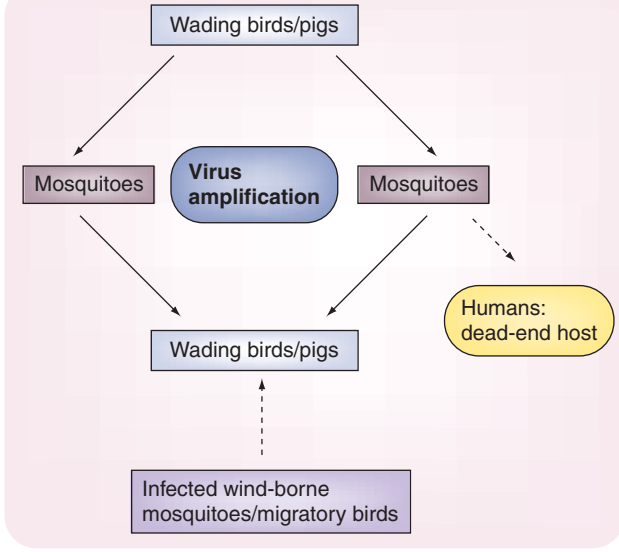

Figure 1. Transmission cycle of Japanese encephalitis virus in nature.

including Japan, China, Taiwan, the Philippines and the Asian subcontinent. Genotype IV includes some isolates from Indonesia. In addition, based on phylogenetic evidence, the Muar strain of JEV isolated in Singapore in 1952 from a patient who originated in Muar (Malaysia), may represent a fifth genotype [26]. These strain variations may have practical implications for vaccine development (see below).

Need for Japanese encephalitis vaccination

JE is the single-most important cause of viral encephalitis in Asia. Although disease incidence is grossly under-reported, WHO estimates indicate that in endemic areas, annual incidence of clinical disease ranges from ten to 100 per 100,000 people, with case fatality averaging 30%. The situation is aggravated by the fact that a high proportion of the survivors, mostly young children, are left with permanent neuropsychiatric sequelae. There is no effective drug treatment for the disease. Moreover, there is no effective method of environmental control of JEV transmission. Since JE is a zoonotic disease with natural reservoirs for the causative agent, it cannot be totally eliminated. Although improvements in agricultural practices due to the betterment of socioeconomic status has led to reduced disease burden in some countries, large-scale vaccination of susceptible human populations appears to be the logical approach towards controling JE, both now, and in the future. The effect of mass vaccination against JE, that has already shown great promise in various regions of China and has led to decreased disease burden in countries such as Japan, Korea and Thailand, provides ample justification for vaccination.

Current vaccines against Japanese encephalitis

Three types of JE vaccines are currently in use of which the mouse brain-derived inactivated vaccine is produced commercially and distributed internationally. The status of these vaccines is provided below.

Mouse brain-derived inactivated Japanese encephalitis vaccine

The mouse brain-derived formalin-inactivated JE vaccine is the only vaccine that is currently WHO approved. Crude preparations of the vaccine had been developed as early as the 1930s in Japan. In modern times, the manufacturing process has been streamlined, which includes virus purification steps. Although most manufacturers still use the original Nakayama strain isolated from a human case in 1935, many have switched to the Beijing-1 strain due to its greater geographical cross reactivity. The BIKEN vaccine manufactured by the Research Foundation for Microbial Diseases of Osaka University in Japan is the most widely used variety of the JE vaccine, while Green Cross (Korea) contributes a smaller share to the global market [27]. Although the BIKEN vaccine continues to be manufactured in Japan, this US FDA-approved vaccine is presently commercially distributed in the USA by Aventis-Pasteur under the trade name JE-VAX®. This vaccine is also independently manufactured in China, India, Taiwan and Thailand. In India, the Central Research Institute, Kasauli manufactures the mouse brain-derived JE vaccine. The vaccine is available in a lyophilized form that is stabilized with gelatin, sodium glutamate and preserved with thimerosal. The current vaccine contains less than 50 ng/ml of mouse serum protein, has undetectable levels of myelin basic protein (detection limit, 2 ng/ml), and is stabilized with 500 mg/ml of gelatin.

Although mouse brain-derived JE vaccines, based on Nakayama or Beijing-1 strains, generate cross-Nt antibodies and exhibit cross protection [28], vaccines made from indigenous strains belonging to particular countries should potentially generate higher levels of protective immunity. However, this may be impractical from the logistical and process economics standpoints.

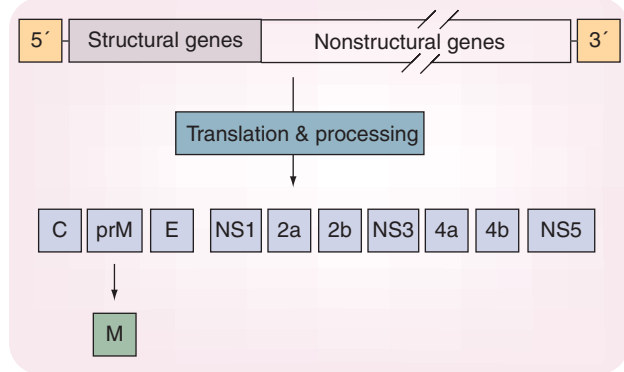

Figure 2. An oversimplified representation of the Japanese encephalitis virus (JEV) genome. The genome is a single-stranded, plus-sense RNA molecule containing a long open reading frame encoding the viral polyprotein with 5' and 3' untranslated regions. See text for a detailed discussion of the structural and nonstructural components of the JEV genome.
E: Envelope; M: Membrane protein; NS: Nonstructural; prM: Premembrane protein.

In a large-scale clinical trial in Thailand involving 65,224 children, two doses of mouse brain-derived inactivated vaccine based on the Nakayama or Beijing-1 strain was found to have an efficacy of 91% [29]. The mouse brain-derived inactivated vaccine was found to be relatively safe. However, common side effects were observed, primarily in vaccinated Asian children and western adults. Local reactions such as erythema, swelling and tenderness have been observed in up to 23% of vaccinees, whereas mild systemic side effects such as fever, headache, malaise and dizziness can be seen in up to 13% of the vaccinees [30]. Moreover, neurological complications, including several cases of acute encephalitis temporally linked to JE vaccination, have been reported. For example, from the Republic of Korea, three cases were reported, of which two were fatal. Furthermore, hypersensitivity reactions such as serious generalized urticaria, facial angioedema and respiratory distress have been observed in adult western vaccinees [31]. Gelatin, which is used as a stabilizer in the vaccine, has been incriminated for these adverse reactions [32].

Owing to its relatively high cost of production, lack of long-term immunity and risk of allergic reactions, large-scale vaccination with the mouse brain-derived inactivated vaccine does not appear feasible, especially in the economically weaker Asian countries, where it is needed most.

Cell culture-derived inactivated Japanese encephalitis vaccine

In recent years, attempts have been made to move away from the mouse brain and use mammalian cells for developing inactivated JE vaccines. A formalin-inactivated JE vaccine is in use, exclusively in China, which is prepared using the Beijing P-3 strain of JEV grown in primary hamster kidney (PHK) cells. The vaccine is relatively easy to manufacture, as it does not involve any purification steps. This is one of the reasons why it has not thus far been licensed in western countries. The manufacturers recommend two doses, followed by a booster dose. Extensive randomized field trials in China, involving nearly half a million infants and children, have shown vaccine efficacy to range between 76 and 95%. Very few side effects of the PHK-derived JE vaccine have been reported. Approximately 4% of the vaccinees have reported local reactions such as erythema and swelling at the injection site, while 1% have reported mild systemic symptoms such as headache and dizziness [33].

The vaccine is inexpensive and approximately 70–90 million doses are distributed annually within China. Lately this vaccine is increasingly being replaced by the SA 14–14–2 live-attenuated JE vaccine, which is even more cheaper to produce and probably more efficacious [34].

Cell culture-derived live-attenuated Japanese encephalitis vaccine

A live JE vaccine produced in PHK cells, involving the stable neuroattenuated SA 14–14–2 strain of JEV, has been licensed for use in China since 1988 [35]. More than 200 million doses of this vaccine have been used in China with a good record of safety and efficacy [36–38]. Immunogenicity studies in children have shown antibody responses in 92% of the vaccinees, and all retained detectable Nt antibodies for 3 years post primary immunization. Protective efficacy was approximately 98% [33]. The success of this vaccine in China has attracted international interest and it has now been licensed in Korea [39]. However, the main hurdle for this vaccine to be adopted by other countries is the PHK substrate, which is not approved by the WHO for human vaccine production.

Japanese encephalitis vaccines under development

In the past 10 years, there has been a rapid explosion in knowledge of the JEV molecular biology and the science of vaccine development. As a result of this, several potential second- and third-generation vaccines against JE have been in development. The immunological basis of protection against JEV infection appears to predominantly involve the virus Nt antibodies, although the role of cellular immune responses cannot be ruled out, and new evidence suggests that the latter's contribution might prove to be more than previously envisaged. Hence, new generation vaccines should ideally induce both of these types of protective immune responses against JEV. On the following pages the authors summarize the recent developments in this field and evaluate different systems and their potential to provide an ideal JE vaccine that may be cheap, safe and capable of providing long-lasting protective immunity.

Vero cell culture-based Japanese encephalitis vaccines

Due to the undesirable side effects of the mouse brain-grown JE vaccine, efforts are being directed towards the development of tissue culture-based vaccines that are likely to be free from the deleterious effects of the former. Various laboratories have focused their attention on Vero cells, derived from the African green monkey (*Cercopithicus aethiops*) kidney to propagate the vaccine virus. Over the last 10 years, more than 100 million doses of inactivated polio vaccine, 20 million doses of rabies vaccine and more than 1 billion doses of live polio vaccine produced in Vero cells have been used worldwide with remarkable records of safety, making Vero a safe cell line for producing vaccines for human use [40].

Several research groups around the world are working on the development of a Vero cell-derived JE vaccine using various local isolates of JEV. Using an attenuated derivative of the Chinese SA 14–14–2 strain of JEV grown on Vero cells, a purified, formalin-inactivated vaccine was made which induced high titers of JEV Nt antibodies in mice in a dose-dependent manner after two injections [41]. The vaccine protected mice against morbidity and mortality after challenge with virulent JEV. Compared with the mouse brain-derived vaccine, the Vero cell-derived, JE-purified, inactivated vaccine was more immunogenic and as effective at preventing encephalitis in mice.

In another development, using the Beijing-1 strain of JEV grown in Vero cells, an industrial-scale process conforming to the current good manufacturing practice (cGMP) criteria has been developed [42]. In the preclinical and Phase I clinical trials

in Japan, the vaccine was found to be safe and its effectiveness to be equivalent to that of the licensed mouse brain-derived vaccine [43]. However, clinical trials using a larger sample size are required to further validate the safety and effectiveness of this vaccine.

The authors have developed a Vero cell-derived, formaldehyde-inactivated JE vaccine using P20778, an Indian strain of JEV [44]. The studies indicated that virus inactivation with formalin at 22°C, which required a shorter incubation period, was as good or better than virus inactivation at 4°C, for it generated higher titers of anti-JEV antibodies in mice. Significantly, sera from the immunized mice effectively neutralized different strains of JEV, albeit with different efficiency. Efforts are currently underway to scale-up this technology for industrial-scale production of this vaccine under the cGMP guidelines.

Recombinant protein-based Japanese encephalitis vaccines

The E protein of JEV and other flaviviruses are involved in such important functions as receptor binding and membrane fusion and has been shown to induce virus-Nt antibodies. As the virus-Nt antibodies alone are considered sufficient for protection against JEV infection, the JEV E protein, thus, has the potential to be used as an immunogen capable of generating protective immunity. The JEV E protein has been synthesized in various forms using different expression systems. Immunogenicity of these various forms of the protein has been tested in a murine model, the details of which are presented below.

Japanese encephalitis virus envelope protein synthesized in the Escherichia coli *expression system*

Early studies failed to localize the neutralization antigenic site in the linear amino acid sequence of JEV E protein, but eventually parts of the E protein were synthesized in an *Escherichia coli* expression system [45]. A 27-residue-long fragment, between amino acids 373 and 399 of JEV E protein, fused to protein A or glutathione-*S*-transferase, used in conjunction with a strong adjuvant elicited virus-Nt antibodies in mice [46]. The same peptide generated JEV-Nt antibodies in mice when presented using the Johnson grass mosaic virus-like particles without the use of an adjuvant [47]. Although the immunized mice were protected against lethal JEV challenge, utility of this vaccinogen, comprising a single epitope, is very limited.

In yet another study, the domain III fragment of the JEV E protein, comprising amino acids 303–396, was expressed as a thioredoxin fusion protein in *E. coli* [48]. The fusion protein used with the Freund's adjuvant or cationic liposome preparation, elicited Nt antibodies that protected the mice against lethal JEV challenge.

The authors have recently evaluated the possibility of developing an oral vaccine against JEV [49]. Recombinant E protein synthesized in *E. coli* was administered orally to mice with an immunostimulatory cytosine–phosphate–guanosine (CpG) motif containing oligodeoxynucleotide as an adjuvant. The immunized mice produced high titers of anti-E and -JEV antibodies that, however, failed to neutralize JEV activity, and did not protect the mice against lethal JEV challenge. The absence of Nt antibodies may be related to the use of the denatured E protein. These results, however, demonstrated the oral immunogenicity of the JEV E protein, suggesting that a properly folded protein may generate antibodies with Nt activity.

Japanese encephalitis virus proteins synthesized in the baculovirus expression system

The baculovirus expression system, utilizing *Autographa californica* nuclear polyhedrosis virus (AcNPV), has been used to obtain expression of many foreign genes, including those that require proteolytic processing, glycosylation or secretion. A major advantage of this system is the abundant expression of recombinant proteins, which are, in many cases, antigenically, immunogenically and functionally similar to their native counterparts.

A recombinant baculovirus containing the complete coding sequence of JEV structural proteins prM and E, together with the parts of sequences encoding the C and the NS1 proteins, was found to synthesize the processed prM and the E proteins in *Spodoptera frugiperda* (*Sf*)-9 cells [50]. The E protein synthesized by the baculovirus recombinant was glycosylated and similar in size to the authentic viral protein, and was located on the surface of the infected cells. Mice immunized with cells infected with the recombinant viruses developed JEV-Nt antibodies, although the titers were lower than those seen in JEV-infected mice. Protective efficacy of the recombinant baculovirus-expressed E protein was, however, not investigated.

In another study, baculovirus recombinants were constructed to synthesize the E or the NS1 glycoproteins of JEV individually or together [51]. Approximately 70% protection was recorded in mice immunized with cells infected with recombinant virus synthesizing the E or the E + NS1 proteins, compared with the 30% protection seen in the unimmunized mice. No protection was seen in mice immunized with cells infected with the NS1-expressing recombinant. Nt antibodies were detected only in E glycoprotein-recipient mice. The significance of the protection reported in these studies is not clear, as the virus dose used for the challenge was suboptimal, since only 70% of the control mice succumbed to the challenge. However, in a recent study the baculovirus-expressed JEV E protein was found to be more efficacious [52]. In this study, 100% of mice immunized with *Sf*-9 cells infected with the recombinant baculovirus synthesizing the prM and E proteins, were protected from a lethal JEV challenge, while only 8% of mice in the unimmunized control group survived the challenge. This study thus provides encouraging evidence for pursuing further work on baculovirus systems for developing a protein subunit vaccine against JEV.

Particulate Japanese encephalitis virus immunogens synthesized in cell culture-based expression systems

Particulate immunogens, dubbed virus-like particles (VLPs), subviral particles (SVPs) or extracellular particles (EPs), have been expressed in bacteria or different mammalian cells. Using

E. coli as the host, the authors produced VLPs of Johnson grass mosaic virus presenting peptides from JEV E protein [47]. These VLPs presenting a 27-amino acid peptide representing amino acids 373–399 from the JEV E protein induced Nt antibodies that protected the mice against a lethal JEV challenge.

The full-length JEV E protein when expressed in mammalian cells remained within the cell in a form that was poorly immunogenic. However, when it was expressed together with prM, as a prM–E fusion protein, it was secreted as SVPs that were highly immunogenic in mice inducing JEV Nt antibodies and virus-specific memory T lymphocytes which conferred protection against live virus challenge [53,54]. Chinese hamster ovary (CHO), COS-1 and rabbit kidney-derived RK13 cells, permanently transfected with the JEV prM–E sequence showed continuous production of EPs which generated Nt antibodies in immunized mice that protected them against lethal JEV challenge [55–57]. This particulate JEV antigen has the potential to be a promising second-generation JE subunit vaccine that should be safe, cheap and effective.

Recombinant virus-based Japanese encephalitis vaccines

Recombinant viruses are useful tools in vaccine research. A variety of viruses have been investigated as potential vectors for developing recombinant viruses. Although each viral vector has its own unique characteristics, an important feature of almost all recombinant viruses is their ability to induce both humoral as well as cell-mediated immune responses. A major advantage of using viral vectors is the fact that the antigen-encoding genes within the recombinant virus are amplified along with the normal replication cycle of the virus, thus increasing the antigen exposure to the immune system. Besides, foreign antigens are processed and presented to the immune system in a manner resembling natural infection. Canarypox, vaccinia, YF and more recently, adenoviruses have been used for the development of recombinant vaccines against JE. These are briefly reviewed below.

Recombinant poxviruses synthesizing Japanese encephalitis virus proteins

In 1991 Konishi and colleagues constructed vaccinia recombinants expressing different JEV proteins. They showed that vaccinia recombinants coexpressing the structural proteins prM and E generated high titers of JEV Nt antibodies in mice which were protected against a lethal JEV challenge. However, recombinants expressing NS1 generated anti-NS1 antibodies, although these induced only a low level of protection in mice against lethal JEV challenge [58]. Subsequently, in 1993 Jan and colleagues showed that a vaccinia recombinant synthesizing a C-truncated JEV E protein had enhanced immunogenicity and protective efficacy in mice [59].

Safety concerns associated with the vaccinia virus have led to the development of highly attenuated derivatives of it. NYVAC is one such virus derived from vaccinia by the deletion of 18 ORFs [60]. Immunization of pigs with recombinant NYVAC expressing JEV prM, E and NS1 proteins generated JEV Nt antibodies which were elevated further, following a booster dose [61]. The immunized pigs developed lower levels of viremia (serum JEV titers) compared with the nonimmunized controls, when challenged with a high dose of live JEV.

Another attenuated derivative of vaccinia that has been used for making recombinant viruses for vaccine purpose is the modified vaccinia virus Ankara strain (MVA). The MVA/JEV recombinants generated JEV Nt antibodies comparable with those induced by the commercial inactivated JEV vaccine in mice and these were protected against a highly lethal dose of JEV [62].

Due to the safety concerns associated with replication-competent recombinant viruses, avipoxviruses have been examined as potential recombinant vaccine vectors. These viruses infect mammalian cells abortively, while maintaining the capacity to present antigens to the immune system. Canarypox virus has received the most attention as the recombinant vaccine vector since it induced immunity more efficiently than other avipoxviruses [63]. ALVAC is an attenuated vaccine strain of canarypox virus. Mice immunized with an ALVAC recombinant expressing the prM, E and NS1 proteins of JEV produced JEV Nt antibodies that protected them from a lethal JEV challenge [64].

The NYVAC–JEV and ALVAC–JEV recombinants have been evaluated in rhesus monkeys where they were found to be safe and immunogenic to varying degrees [65]. While all monkeys (four out of four) immunized with the BIKEN vaccine survived the intranasal JEV challenge, three out of four NYVAC–JEV immunized, and one out of four ALVAC–JEV-immunized monkeys survived. These recombinants were further evaluated in a controlled, randomized, double-blind clinical trial to assess safety and immunogenicity in human volunteers [66]. Groups of vaccinia-immune or vaccinia-naive individuals, comprised of ten volunteers each, were given two doses of each recombinant vaccine. The recombinant vaccines were equally safe and well tolerated, but more reactogenic than the commercial formalin-inactivated JE vaccine. The vaccinees experienced frequent occurrence of local warmth, erythema, tenderness and/or arm pain after vaccination. There was no apparent effect of vaccinia immune status on the frequency or magnitude of the local and systemic reactions. NYVAC–JEV elicited antibody responses to JEV antigens in recipients, but here also, as in the previous study in rhesus monkeys, ALVAC–JEV vaccine was poorly immunogenic. However, the NYVAC–JEV vaccine induced Nt antibodies only in the vaccinia-naive recipients, while vaccinia-immune volunteers failed to develop protective antibodies. These data indicated that pre-existing immunity to poxvirus vector interfered with the antibody responses to the recombinant gene products suggesting that alternate viral vectors for antigen delivery need to be investigated.

Recombinant adenovirus synthesizing Japanese encephalitis virus proteins

In recent years, adenoviruses have shown great promise as vectors for recombinant vaccine development. Besides being safe, these viruses have been shown to induce effective humoral

and cellular immune responses in experimental animals. In ongoing efforts to develop potential JEV vaccine candidates, the authors constructed a recombinant virus using human adenovirus 5 (Ad5) that synthesized the prM and E proteins of JEV [67]. Recombinant adenovirus (RAdEs) synthesizing the C-truncated secretory form of JEV E protein was significantly more immunogenic in mice than the recombinant synthesizing the full-length membrane-anchored E protein. Mice immunized with RAdEs, given intramuscularly (IM), generated high titers of JEV Nt antibodies and splenocytes from these animals secreted large amounts of interferon in the presence of JEV and showed cytotoxic activity against JEV-infected cells. Naive mice immunized with RAdEs were completely protected against a lethal intracerebral (IC) challenge with JEV. Mice with pre-existing Ad5 Nt antibody titers similar to those in young children could also be immunized successfully with RAdEs, demonstrating its potential as a candidate vaccine for children, the primary targets of JEV infection.

Recombinant yellow fever virus expressing Japanese encephalitis virus proteins

YF17D is a live, attenuated vaccine that has been in use for nearly 70 years with an excellent record of safety and efficacy. Inoculation of a single dose of YF17D leads to generation of life-long immunity in nearly 100% of the vaccinees. The vaccine manufacturing procedure is well established, and the vaccine is licensed for human use by the international health authorities. Thus, YF17D is an ideal vaccine vector for making recombinant vaccines.

A chimeric virus (ChimeriVax-JE) has been constructed by replacing the genes encoding prM and E proteins of YF17D virus with the corresponding genes of SA 14–14–2, an attenuated strain of JEV [68]. Mice immunized with a single dose of ChimeriVax-JE vaccine demonstrated 100% protection when challenged with a lethal dose of JEV administered intraperitoneally (IP) [69]. Rhesus monkeys immunized with a single dose of the vaccine developed JEV-Nt antibodies. None of the immunized monkeys, when challenged with wild-type JEV administered IC, developed viremia or illness and had mild residual brain lesions, whereas sham immunized control monkeys developed viremia, clinical encephalitis and severe histopathological brain lesions [70].

ChimeriVax-JE can be grown to high titers in diploid fetal rhesus lung (FRhL) cells, which are acceptable substrates for human vaccine production. Extensive safety studies on FRhL-grown ChimeriVax-JE have been carried out in mice and monkeys where it passed the preclinical safety and efficacy requirements for a human vaccine [70]. The molecular basis for attenuation of the ChimeriVax-JE construct was dissected out using site-directed mutagenesis by which one or multiple SA 14–14–2-specific determinants were reverted to the wild-type residues. Neurovirulence studies showed that reversion of three or four amino acids was required to restore the mouse neurovirulence typical of the wild-type JEV. This finding, coupled with the established observations that the SA 14–14–2-specific residues are stable during sequential passage, indicate that the risk of reversion to the virulent wild-type phenotype was exceedingly remote [71].

A randomized, double-blind clinical trial demonstrated that ChimeriVax-JE induced JEV-Nt antibodies in 100% of naive (n = 6) as well as YF17D-immune (n = 6) subjects [72]. A standard plaque reduction neutralization test with a 50% end-point ($PRNT_{50}$) was employed to detect the level of Nt antibodies in the sera of the subjects. JE antibody levels were higher in YF-immune than in naive subjects, dispelling concerns about the antivector immunity. Subsequently, in a double-blind Phase II clinical trial carried out in a larger number of individuals (n = 99) who received either ChimeriVax-JE, placebo or the YF17D vaccine, ChimeriVax-JE was found to be well tolerated with 94% of individuals developing JEV-Nt antibodies [73]. Significantly, immunological memory was demonstrated in ChimeriVax-JE-immunized individuals in the form of an anamnestic response following the challenge with the licensed JE-VAX.

Plasmid DNA-based Japanese encephalitis vaccines

Nucleic acid or genetic vaccines, more popularly known as DNA vaccines, represent the most recent of the vaccine development strategies. In this approach, DNA encoding the protective immunogen, or a part thereof, is placed under the control of a strong eukaryotic promoter in a bacterial plasmid which is then administered IM or intradermally (ID). The plasmid incorporating the antigen-encoding DNA fragment is endocytosed and endogenous protein synthesis allows the presentation of the foreign antigen by the major histocompatibility complex (MHC) class I, thereby generating a $CD8^+$ cytotoxic T-lymphocyte (CTL) response. Furthermore, uptake of soluble proteins by specialized antigen-presenting cells (APC) allows presentation by MHC class II, thereby generating a $CD4^+$ helper T-cell response. DNA vaccines offer potential advantages over other modes of vaccination. For example, vaccine interference due to pre-existing antibodies to other flaviviruses or to the vaccine vector is not a problem with DNA vaccines. Besides, these vaccines are likely to be cheaper, safer and easier to manufacture, making them an attractive alternative to conventional vaccines. Over the past 10 years, quite a few candidate DNA vaccines for JE have been developed and tested in animal models with varying degrees of success. These are reviewed below.

DNA vaccines expressing Japanese encephalitis virus structural proteins

It has been known that the antibodies directed against JEV E protein neutralize virus activity *in vitro* and the JEV-Nt antibodies are important mediators of protection against the disease. Moreover, it has been established that another structural protein, prM, is essential for the intracellular processing and secretion of the E protein in the correct conformation. As a result, the majority of JEV DNA vaccine constructs incorporate both E and prM.

Early studies with JEV DNA vaccine candidates encoding prM and E genes demonstrated that it elicited low titers of Nt antibody in mice with 70% of the mice surviving a lethal challenge [74]. At least two doses of vaccine were required to induce Nt antibodies. Spleen cells of the immunized mice had JEV-specific CTLs and memory B cells that persisted for at least 6 months. It was observed that this DNA vaccine candidate was more immunogenic in pigs than the formalin-inactivated veterinary version of JE-VAX, and that the Nt antibody titers persisted for almost 8 months [75]. Subsequently, Chang and colleagues demonstrated that a single IM injection of as little as 25 µg of recombinant plasmid DNA encoding the JEV prM and E proteins, induced JEV Nt antibodies which protected the immunized mice against lethal viral challenge [76]. Besides, passively transferred Nt antibodies from the plasmid DNA-immunized mother protected young pups against lethal JEV infection. These remarkable findings stem from the novel design of the gene cassette that included the Kozak's consensus sequence for enhanced translation and the computer-predicted optimal signal peptide-encoding sequence preceding the prM and E genes of JEV.

Ashok and Rangarajan evaluated the immunogenicity of a plasmid that contained the JEV E-encoding sequence without any signal peptide [77]. The E protein synthesized by the plasmid remained intracellular. The plasmid was able to raise a protective immune response in mice even though JEV-specific antibodies were undetectable. In another study, mice immunized with the plasmid synthesizing JEV E protein along with the signal sequence from the C-terminus of the M protein, generated anti-JEV antibodies in mice, yet they did not have detectable levels of JEV Nt activity. However, these mice were protected against a lethal JEV challenge [78]. Since these plasmids did not encode prM necessary for the proper folding of JEV E protein, the E protein generated might have had a non-native conformation, thereby explaining its inability to generate detectable amounts of Nt antibodies. The protection observed in mice may perhaps be due to the priming by the DNA vaccine and the subsequent memory B-cell response following the challenge. The importance of this anamnestic response in the survival of the immunized animals has been reiterated in another study where mice that developed high titers of JEV Nt antibodies after challenge were shown to be more likely to survive than those that did not [79].

Considering that the form of the immunogen might affect its immunogenicity, the authors studied the immunogenicity in mice of plasmids, delivered IM or ID using a gene gun, that synthesized the membrane-anchored and the secreted forms of the JEV E protein [80]. The form of the E protein or the route of DNA delivery did not affect the level of protection seen in immunized mice where approximately 60% protection was seen in an IC JEV challenge model. This was significantly lower than approximately 90% protection afforded by the commercial formalin-inactivated vaccine in mice.

DNA vaccines expressing Japanese encephalitis virus nonstructural proteins

In addition to the structural proteins, the *Flavivirus* nonstructural protein NS1, in spite of having no recognizable membrane anchor sequence, is expressed on the surface of infected cells, besides being secreted in the extracellular environment [81]. The NS1 protein of YF virus has been shown to elicit a protective immune response in mice [82]. Plasmid DNA expressing JEV NS1 protein has also been shown to elicit protective immunity in mice [81]. The NS1 plasmid immunization elicited a strong antibody response exhibiting cytolytic activity against JEV-infected cells in a complement-dependent manner, in spite of the fact that no Nt activity could be detected. The construct expressing a longer NS1 protein (NS1´), containing an extra 60 amino acid portion from the N-terminus of NS2A, failed to protect mice against a lethal JEV challenge [78,81]. Studies revealed that individually expressed NS1, but not NS1´, was readily secreted as a homodimer in large quantities, besides being expressed on the cell surface. This perhaps made it significantly more immunogenic than NS1´ which was likely to be retained in the endoplasmic reticulum rather than proceed along the secretory pathway.

The *Flavivirus* nonstructural proteins NS3 and NS5 are involved in viral genome replication. It has been observed that the cell-mediated immune responses are directed mainly against the conserved nonstructural viral proteins. The NS3 and NS5 proteins are highly conserved amongst flaviviruses. However, plasmids encoding JEV nonstructural proteins NS3 and NS5 were found to be ineffective in raising protective immunity [78]. In another study, immunization of mice with plasmids encoding nonstructural proteins NS1, NS2A, NS2B, NS3 or NS5 induced CTL responses that provided only partial protection against an IP challenge with a lethal dose of JEV, whereas complete protection was observed in mice immunized with the plasmid encoding prM and E proteins [83]. This study indicated that the protection afforded by the prM/E-encoding DNA consisted of Nt antibodies that prevented virus dissemination from the peripheral site to the brain and that this antibody-mediated mechanism of protection was more efficient than the immunity induced by the plasmids encoding the nonstructural proteins that induce CTL responses. This observation strengthens the earlier conclusion that JEV-Nt antibody is the most critical protective component whose production is dependent on the presence of $CD4^+$ T cells but independent of $CD8^+$ T cells [84].

Encouraged with the potential of DNA vaccines against JE, efforts are now focused on improving their immunogenicity. The authors have tried to address this issue in two ways. First, the authors have investigated the delivery of plasmid DNA in a particulate form to enhance its uptake by the professional APCs. The authors found that DNA adsorbed onto cationic microparticles demonstrated enhanced immunogenicity in mice [85]. The authors believe

that use of smaller cationic particles in the nano range would further enhance the efficacy of the candidate DNA vaccine. Indeed, mice immunized with prM and E-encoding plasmid adsorbed onto colloidal gold produced JEV-specific Nt antibodies that protected them against a highly lethal dose of JEV [86]. Second, the authors have investigated whether coadministration of JEV DNA vaccine and plasmids encoding the cytokines interleukin (IL)-2 or granulocyte–macrophage colony-stimulating factor (GM-CSF) in mice improved the immunogenicity of the DNA vaccine. The authors findings showed that ID coadministration of JEV DNA vaccine and plasmid-encoding GM-CSF using the gene gun enhanced anti-JEV antibody titers resulting in an increased level of protection in mice against lethal JEV challenge [87]. In addition, newly developed adjuvants such as vaxfectin have been used to enhance the immunogenicity of JEV DNA vaccine candidates [88]. Besides, *in vivo* electroporation of skeletal muscles was shown to enhance the immunogenicity of a JEV DNA vaccine in mice, although the technique may have practical limitations [89].

Expert commentary & five-year view

Despite the immense clinical significance of flaviviruses in general and JE in particular, there is, as yet, no chemoprophylactic or chemotherapeutic agent available for these viruses. Although there is some hope that antiviral agents may be found in the future, for the present, vaccination appears to be the only logical alternative. However, it should be borne in mind that JE cannot be eradicated fully as for diseases such as smallpox, polio or measles, where humans are the only host. At most, this zoonotic disease can be held at bay by the judicious application of vaccination of humans.

Now that we are slowly moving away from the mouse brain and exploring other avenues of JE vaccine production, it is a good sign for the coming years. The two main factors that need to be addressed carefully while investigating the applicability of some of these alternative vaccines are their long-term efficacy and cost. Policy makers and health officials should bear in mind that the target populations for JE vaccines are often unable to afford high cost vaccines. This is an important reason why the mouse brain-derived vaccine could not be used for mass-scale vaccination.

At present we have quite a few promising JE vaccine candidates that are in various stages of development. Vero cell-derived inactivated JEV vaccine and YF virus-based recombinant vaccine ChimeriVax-JE are under clinical evaluations. JEV DNA vaccine has been evaluated in nonhuman primates and may soon enter clinical trials [90]. The coming 5 years, thus, will be an exciting period for JE vaccine development where an efficacious vaccine is likely to emerge. However, further research efforts need to be directed towards making these as single-dose vaccines with a long-lasting immune response, since, vaccines that could be administered by noninvasive means would be most desirable.

Although the main body of evidence lays stress on the Nt antibody response as the yardstick for protection against JEV, at least two reports indicate that the cellular responses might also be relevant for protection [77,78]. It may, therefore, be important to investigate the human immune response relevant for protection against JEV. In fact, a recent study has addressed this issue by studying cell-mediated immune responses in healthy children with a history of subclinical JEV infection [91]. This study demonstrates that nonstructural proteins are frequently targeted by T cells in natural JEV infections and these may be efficacious supplements for the predominantly antibody-eliciting E-based JEV vaccines.

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Key issues

- Japanese encephalitis (JE) caused by the *Flavivirus* JE virus (JEV) is a serious disease with high rates of mortality and morbidity. The disease is prevalent in most parts of Asia and has the potential to spread to hitherto unaffected areas such as the Australian continent.
- A mouse brain-derived JE vaccine is currently available internationally which is effective, but is expensive and not completely safe. Thus, there is a need for an alternate vaccine that may be cheaper, safer and effective over a long duration.
- An attenuated JE vaccine based on the SA 14–14–2 strain of JEV is in use in the People's Republic of China. This vaccine is produced in primary hamster kidney cells that are not approved for human vaccine production by the WHO.
- Several candidate JE vaccines are in different stages of research and development. These include Vero cell-cultured inactivated JE vaccine, chimeric yellow fever/JEV vaccine and DNA vaccines.
- JE cannot be completely eradicated, as it is a zoonotic disease. However, mass-scale vaccination of the human population can significantly reduce the disease incidence. Also, surveillance and vaccination should be recommended in countries near the traditional boundaries of JEV activity.

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