Japanese encephalitis (JE) is an infectious disease of the central nervous system caused by Japanese encephalitis virus (JEV), a zoonotic mosquito-borne flavivirus. JEV is prevalent in much of Asia and the Western Pacific, with over 4 billion people living at risk of infection. In the absence of antiviral intervention, vaccination is the only strategy to develop long-term sustainable protection against JE infection. Over the past half-century, a mouse brain-derived inactivated vaccine has been used internationally for active immunization. To date, however, JEV is still a clinically important, emerging, and re-emerging human pathogen of global significance. In recent years, production of the mouse brain-derived vaccine has been discontinued, but 3 new cell culture-derived vaccines are available in various parts of the world. Here we review current aspects of JEV biology, summarize the 4 types of JEV vaccine, and discuss the potential of an infectious JEV cDNA technology for future vaccine development.

Ecology and Epidemiology

Japanese encephalitis (JE) is an inflammatory disease of the brain caused by Japanese encephalitis virus (JEV). JE is the most common cause of viral encephalitis in Asia, from the China-Russia border region in the north to the northern Australia in the south, and from the Western Pacific islands in the east to the India-Pakistan border region in the west (Fig. 1).

Histologically, JE-like outbreaks were recorded in Japan in the late 1800s, but the first confirmed JE case was reported in Japan in 1924, followed by Korea (1933), China (1940), the Philippines (1950), India (1955), and a number of other Asian countries thereafter. Over the past few decades, JE incidence has decreased considerably in some countries (e.g., Japan, South Korea, and Taiwan) but has increased in others (e.g., Bangladesh, Cambodia, India, Indonesia, and Pakistan). In the late 1990s, JEV began to emerge in the Torres Strait islands and spread onto the Cape York Peninsula, posing a serious risk to public health in Australia and raising a significant concern that the virus may continue to spread throughout the world.

Transmission

JEV is an arthropod-borne virus (arbovirus) that is transmitted in an enzootic cycle among mosquito vectors and vertebrate hosts, particularly pigs and birds; and humans become infected when bitten by an infected mosquito (Fig. 2). Although many
manifestations ranging from undifferentiated febrile illness to acute encephalitis. The prodromal phase of the disease begins with flu-like non-specific symptoms, including fever, headache, malaise, and vomiting, that may last for several days. This mild febrile illness is followed by the acute encephalitic phase, in which a variety of neurological symptoms manifest themselves (e.g., mental status changes, focal neurologic deficits, and movement disorders). JE patients often show a parkinsonian syndrome, which is characterized by tremor, cogwheel rigidity, and hyperpnea. Also, a significant proportion of JE patients experience polio-like acute flaccid paralysis. Convulsions and abnormal behavior are common in children, whereas febrile illness and meningism occur frequently in adults. The most common complications associated with a poor prognosis include persistent seizures, motor neuron weakness, cerebellar signs, extrapyramidal disorders, arm flexion deformities, leg hypertensions, cognitive deficits, language impairments, learning disabilities, and behavioral problems.

Virology

Genome structure and gene expression
JEV is a member of the genus *Flavivirus* in the family *Flaviviridae*. Within the genus, JEV is the prototype virus of the JE serogroup, which also includes several medically important etiological agents of encephalitis, such as West Nile virus, St. Louis encephalitis virus, and Murray Valley encephalitis virus. Taxonomically, JEV is closely related to other clinically important flaviviruses, including yellow fever virus (YFV), dengue virus, and tick-borne encephalitis virus (TBEV). Like all flaviviruses, JEV is a small enveloped virus, ~50 nm in diameter, with a single-stranded positive-sense RNA genome that has a 5′-type I cap but lacks a 3′-terminal poly(A) tract. The genome encodes a single long open reading frame (ORF) flanked by 2 short non-coding regions (NCRs) at the 5′ and 3′ ends. In the case of JEV CNU/LP2, a genetically well-characterized virulent strain, the genomic RNA is 10968 nucleotides in length, consisting of a 95-nucleotide 5′NCR, a 10299-nucleotide ORF (3432 amino acids plus a stop codon), and a 574-nucleotide 3′NCR.

In flaviviruses, the genomic RNA is equivalent to a functional mRNA. Upon infection into a susceptible cell, the ORF encoded in the genome is translated in a cap-dependent manner into a polyprotein precursor, which is co- or post-translationally cleaved by host and viral proteases into a panel of at least 10 functional proteins, designated C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5 in an N- to C-terminal direction. Of these, the 3 N-terminal structural proteins (C; prM, the precursor of M; and E) are required for the formation of infectious virions. A viral particle has a nucleocapsid, a complex of the genomic RNA with multiple copies of the highly basic C proteins, which is enveloped by a host-derived lipid bilayer containing the 2 membrane-anchored surface proteins, prM/M and E.

Clinical Presentation

JEV has an incubation period of ~5–15 d from the initial exposure to JEV until the appearance of the first symptom. Symptomatic JEV infection can cause a spectrum of clinical manifestations ranging from undifferentiated febrile illness and aseptic meningitis to acute encephalitis. The prodromal phase of the disease begins with flu-like non-specific symptoms, including fever, headache, malaise, and vomiting, that may last for several days. This mild febrile illness is followed by
extended form of NS1 (previously known as NS1') has been reported to be the product of a classical -1 ribosomal frameshifting, which occurs between codons 8 and 9 of NS2A and adds 52 extra amino acids. Site-directed mutagenesis has suggested that NS1' is involved in viral neuroinvasiveness.

Replication cycle

An overview of the flavivirus replication cycle is schematically illustrated in Figure 4. The first step is attachment of the virion to the host cell in a non-specific manner. Subsequently, the viral E protein is believed to bind with high specificity to an unknown cellular receptor(s) on the cell surface. The particles are then internalized by receptor-mediated, clathrin-dependent endocytosis. Upon exposure to the acidic conditions in endosomes, the E protein undergoes conformational changes, which trigger fusion of the viral membrane with the cellular endosomal membrane.

Once the viral genomic RNA is uncoated, it is translated into a polyprotein in the endoplasmic reticulum (ER), which is processed by a combination of host and viral proteases to yield the functional structural and nonstructural proteins. The viral nonstructural proteins, presumably together with a number of host factors, are responsible for the replication of the viral genomic RNA, which takes place in the viral “replication complex” that is associated with the ER-derived membranes.

At the early stage of viral assembly, immature virions are formed by budding of the viral genomic RNA and C proteins into the lumen of the ER, where the prM and E proteins are incorporated into the budding particles. These immature virions are then transported through the cellular secretory pathway. In the trans-Golgi network (TGN), the immature virions are exposed to the low pH environment and undergo structural changes that render the cleavage site of prM accessible to the cellular protease furin. The cleavage of prM to M and the low pH in the TGN induces the maturation of the viral particles, which is also accompanied by significant structural rearrangements of the prM/M and E proteins. Both completely and partially mature virions are secreted from the infected cells.

Vaccines

There are still no specific drugs available to treat JEV infection. In the absence of antiviral therapy, JEV is managed only with supportive therapies and preventive measures. Based on the mode of transmission, the prevention of JEV is based essentially on 4 strategies, i.e., mosquito control, avoiding mosquito bites, pig immunization, and human immunization. Of these, human immunization is the active method of choice to achieve long-term sustainable protection against JEV. Currently, there are 4 different types of JEV vaccines available for humans in various areas of the world (Table 1): (1) mouse brain-derived killed-inactivated, (2) cell culture-derived live-attenuated, (3) cell culture-derived killed-inactivated, and (4) genetically engineered live-attenuated chimeric vaccines.

Mouse brain-derived killed-inactivated vaccines

The first licensed JEV vaccine was an inactivated mouse brain-derived vaccine based on the prototype JEV strain Nakayama, the first isolate recovered from the brain of a JEV patient in 1935 in Japan. Although its purity has been improved over the course of the past ~60 y, the vaccine was typically produced by inoculating suckling mice intracerebrally with the virus, inactivating the supernatant of the infected mouse brain homogenate with formalin, and purifying inactivated virus suspension by ultracentrifugation. No mouse myelin basic protein could be detected in the vaccine at a detection limit of 2 ng/ml. The mouse brain-derived inactivated Nakayama vaccine was marketed as JE-VAX and was the only commercially available vaccine worldwide for several decades.

Similar mouse brain-derived JEV vaccines were produced by multiple manufacturers in India, Japan, Korea, Taiwan, Thailand, and Vietnam. Although highly immunogenic and efficacious, JEV-VAX had several drawbacks and limitations, including vaccine-induced adverse events, high production costs, and the need for 2 or 3 primary doses plus boosters. In addition to a considerable incidence of local and mild systemic side-effects, there was also a risk of rare but serious allergic and neurologic side-effects. It could cause post-vaccination cases of acute disseminated encephalomyelitis, which have also been associated with a number of other vaccines for rabies, smallpox, measles, mumps, rubella, pertussis, influenza, and hepatitis B. In light of the availability of the new cell culture-derived JEV vaccines, production of JE-VAX was halted in 2006, and all the remaining stock expired in 2011.

From 1989 until the mid-2000s, the mouse brain-derived vaccine had been produced in Japan using two JEV strains, the Nakayama for international distribution and the Beijing-1 (P1)
Initially, the Beijing-1 strain was considered to be able to induce a more potent immunogenicity and elicit broader cross-reacting antibodies against heterologous JEV strains. However, several comparative studies showed that both the Nakayama- and Beijing-1-based mouse brain-derived vaccines were equally capable of eliciting high levels of immunogenicity and protective efficacy against a wide range of JEV strains. For decades, the 2 mouse brain-derived vaccines had therefore been used for the effective control of JE in several endemic countries, including South Korea, Japan, Taiwan, and Thailand. In most cases, a 2-dose primary immunization schedule was used, giving the first dose at any age between 1 and 3 years and the second dose 1–4 weeks later; the first booster dose was given at 1 year after primary vaccination, and then repeated boosters were given every 1 to 3 y up to 10–15 y of age.

**Cell culture-derived live-attenuated vaccine**

A live-attenuated cell culture-derived JE vaccine was developed in China based on the SA14-14-2 strain, an attenuated form of the virulent JEV strain SA14 isolated from a pool of *Culex pipiens* mosquito larvae collected in China in 1954. The SA14-14-2 strain was generated by serial passage in primary hamster kidney (PHK) cells and in animals (i.e., mice and hamsters), combined with multiple plaque purifications in PHK or primary chick embryo (PCE) cells during the passages. The live-attenuated vaccine named SA14-14-2 was first licensed for commercial application in China in 1988 and is currently being produced in PHK cells. Since its licensure, >300 million doses of SA14-14-2 have been produced for administration to Chinese children, with an excellent record of safety and efficacy.

Over the past decade, SA14-14-2 has been progressively licensed in other Asian countries, including South Korea, Nepal, India, Sri Lanka, Cambodia, Laos, Myanmar, and Thailand. Today, SA14-14-2 is the most widely used JE vaccine in JE-endemic areas. In China, SA14-14-2 has been administered to children (9–12 mo) in 2 doses 1 year apart; a booster dose is given at school-entry age. To promote progress toward expanding the international licensure of SA14-14-2 outside of Asia, several key issues needed to be addressed that relate to quality control of the adventitious agents in the uncharacterized cells used for vaccine production: (1) maintaining the hamster colonies under specified-pathogen-free conditions; (2) monitoring the vaccine seeds to ensure freedom from adventitious agents; (3) testing batches of the vaccine for attenuated phenotype in suckling mice, weanling mice, and monkeys; and (4) controlling and recording the raw materials (e.g., hamster cells and bovine sera) used to produce the original vaccine seeds.

SA14-14-2 is highly immunogenic, as shown by the high percentage of seroconversion with 1 dose (85%–100%) and near-complete seroconversion with 2 doses given 1 to 3 mo apart. Consistent with its high level of immunogenicity, several case-control studies have indicated that SA14-14-2 is highly efficacious in preventing JE, with a high protection efficacy following 1 dose (80%–99%) and almost complete protection after 2 doses (>98%). A recent follow-up study has shown that in a JE-endemic area of Nepal, a high level of neutralizing antibody is maintained in children vaccinated with a single dose of SA14-14-2 after 4 (~90%) and 5 (~64%) years of vaccination. In Nepal, 2 case-control studies have also provided evidence of sustained high protection elicited by a single dose of SA14-14-2 at 1 (~98%) and 5 (~96%) years after the initial vaccination. However, the durability of protective immunity needs further investigation, because in JE-endemic areas, vaccinated individuals are continuously exposed to natural JEV infection. With respect to vaccine safety, no severe vaccine-induced adverse events have been observed. Thus, SA14-14-2 appears to be effective and safe when administered...
in a 2-dose regimen. Still, there is a theoretical risk for reversion of the attenuated SA14-14-2 virus to high virulence, which has restricted its extended application to global immunization for the prevention of JEV infection.18,154

**Cell culture-derived killed-inactivated vaccines**

A PHK cell-derived inactivated JE vaccine was developed using the Beijing-3 (P3) strain, a Chinese isolate recovered in 1949 from the brain of a patient during the Beijing-1 strain epidemic.18,202 Since 1968, this PHK cell-derived inactivated Beijing-3 vaccine has been widely used in China and was adapted to production in African green monkey kidney (Vero) cells.18,203 In China, the Vero cell-derived Beijing-3 vaccine was licensed in 1998 and is now the leading inactivated vaccine for domestic use, but it is being replaced by the live-attenuated SA14-14-2 vaccine.18,202,204 In Japan, another Vero cell-derived inactivated vaccine was produced using the Beijing-1 strain and is currently available under the two trade names (IXIARO, JESPECT, and JEEV) in many countries, including the US, Europe, Canada, Australia, Hong Kong, Switzerland, and India.51,209-211 In all cases except India, the initial licensure of IC51 is limited to adults aged ≥17 y as a substitute for JE-VAX, which is no longer available.204,212 In India, on the other hand, IC51 is approved for both adults (18 to ≤49 y) and children (1 to <3 y), with the recommendation of 2 primary doses given 28 d apart. In early 2013, the pediatric use of IC51 was also approved for children (2 mo to <17 or 18 y) in the US and Europe. IC51 is formulated with aluminum hydroxide as an adjuvant.209,210 In general, an appropriate adjuvant acts to accelerate, enhance, or prolong the quality of the vaccine-elicited immune responses.216 Aluminum compounds are the most commonly used adjuvants in human vaccination because of their clinical safety, low cost, and strong adjuvanticity with a variety of antigens (albeit with some limitations, such as local reactions, IgE antibody induction, and ineffectiveness to elicit cell-mediated immune responses).214,215 The ideal adjuvant for JE vaccines should enhance the neutralizing antibody response, have the potential for antigen-sparing, and be safe and well-tolerated.216

In adults aged ≥18 y, several clinical trials have indicated that IC51 induces a level of immunogenicity equivalent to or even higher than that of JE-VAX, with a safety and tolerability that have proven to be more favorable.217-221 The good safety profile of IC51 has also been confirmed by post-marketing surveillance, including safety data from 10 phase III clinical trials in >4000 subjects who received at least 1 IC51 vaccination.222 After a
2-dose primary immunization with IC51, the seroprotection rate decreases over time (i.e., 83% at month 6, 58% at month 12, and 48% at month 24), but a booster at 1 or 2 y after the initial vaccination leads to complete seroconversion. Recently, an additional clinical trial has confirmed the long-term immunity following a booster dose of IC51 at 15 mo after the primary immunization. Similarly, in Indian children aged 1 to 3 y, no apparent difference was observed in the immunogenicity and safety profiles of the IC51 and JE-VAX vaccines. Currently, additional clinical trials are ongoing to determine the immunogenicity and safety of the vaccine in children/infants in the US and other countries.

As part of its ongoing efforts to address the interchangeability of JE vaccines, a single dose of IC51 has been shown to be able to boost immunity in subjects primed with JE-VAX, suggesting that two JEV strains, Nakayama and SA14-14-2, are immunologically similar enough to induce a substantial level of cross-reactive immune responses. This finding will have a large impact on heterologous prime-boost vaccination with the Nakayama-based JE-VAX followed by 1 of the 3 SA14-14-2-derived JE vaccines (i.e., SA14-14-2, IC51, or ChimeriVax-JE). Intriguingly, after a single dose of IC51, the JEV-specific neutralizing antibody response is enhanced with pre-existing anti-TBEV immunity, indicating a positive effect of pre-existing immunity against other flaviviruses on the immunogenicity of JE vaccines. Furthermore, a phase III clinical study has reported no influence on the immune response to either JEV or hepatitis A virus (HAV) when IC51 and the HAV vaccine HAVRIX are administered concomitantly to healthy subjects.

**Genetically engineered live-attenuated chimeric vaccine**

A recombinant, live-attenuated JE vaccine based on a chimeric YF-JE virus (designated ChimeriVax-JE) has also been produced; this vaccine is engineered to express the structural prM and E proteins of JEV SA14-14-2 in the context of YFV 17D, a live-attenuated YF vaccine strain. The YFV 17D vaccine was chosen as the ideal vector for production of the chimeric virus because of its excellent record of safety and efficacy for the past 70 y. The ChimeriVax-JE virus is generated by using infectious YFV 17D cDNA technology, in which the genes encoding the prM and E proteins of YFV 17D are replaced with the corresponding genes of JEV SA14-14-2. This Vero cell-derived ChimeriVax-JE vaccine is also referred as IMOJEV, JE-CV, or THAIJEV and is now commercially available in Australia and Thailand. In both countries, 1 dose of the vaccine is recommended for subjects...
Moreover, in mammalian cells, co-expression of the prM and E proteins has been shown to produce a secreted form of subviral particles capable of inducing JEV-neutralizing antibodies and generating JEV-specific cytotoxic T cells in animals.256-259

**Poxvirus-based vaccines**

Recombinant poxviruses have been used as viral vectors to deliver JEV antigens (e.g., prM, E, and/or NS1) that can induce protective immunity in mice.260-263 Three major poxviruses that have been demonstrated the potential for JE vaccine development are NYVAC (an attenuated vaccinia), ALVAC (an attenuated canarypox), and MVA (the modified vaccinia Ankara).257,259,264-266

In a clinical trial, NYVAC- and ALVAC-based JE vaccines were found to be well tolerated, but their immunogenicity did not appear to be satisfactory; in particular, the NYVAC-based vaccine elicited JEV-neutralizing antibodies in vaccinia-nonimmune volunteers but not in vaccinia-immune volunteers, suggesting that pre-existing poxvirus immunity may suppress the induction of immune responses to JEV antigens.267 In addition to these poxviruses, adenoviruses have also been explored as a viral vector to express JEV antigens in animals.268

**Plasmid DNA-based vaccines**

In mice and pigs, immunization of a plasmid encoding the prM and E proteins can induce a range of protective immune responses that include JEV-specific B cells and cytotoxic T cells.256,269-271 In their effort to maximize the immunogenicity and protective efficacy of JEV prM-E DNA immunization, several studies in mice have tested a variety of DNA constructs encoding an intracellular, membrane-anchored, or secreted form of the E protein, with or without the expression of the prM protein.262,272,273 Also, the potential use of cytokine adjuvants has been studied in mice by co-administering a plasmid expressing GM-CSF or IL-12; in both cases, however, the co-inoculation appeared to suppress the cellular and antibody immune responses and protective immunity induced by a JEV DNA vaccine expressing the E, prM-E, or prM-E-NS1 protein.274-276

In addition to the two structural proteins (prM and E), immunization with a plasmid encoding the viral nonstructural protein NS1 can also induce an antibody response with cytolytic activity in a JEV-specific, complement-dependent manner.271,277 The NS1 immunization thus has been shown to be sufficient to protect mice against a lethal infection with JEV, despite having no detectable neutralizing antibodies induced.271

**Vaccine efficacy and JEV genotype replacement**

Over the past 2 decades, there has been a dramatic shift in the dominant genotype of JEV circulating in JE endemic areas.

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**Table 1. Summary of 4 different types of JE vaccines**

| Vaccine type                          | JEV strain               | Vaccine name (Manufacturer)                     |
|--------------------------------------|--------------------------|------------------------------------------------|
| Mouse brain-derived killed-inactivated | Nakayama/Beijing-1 (P1) | JE-VAX (BIKEN)                                  |
| Cell culture-derived live-attenuated  | SA<sub>a</sub>-14-2       | SA14-14-2 (CDIBP<sup>a</sup>)                     |
| Cell culture-derived killed-inactivated | Beijing-1 (P1)           | JEBIK V (BIKEN); ENCEVAC, KD-287, or JEMMUGEN INJ (Kaketsuken) |
|                                      | Beijing-3 (P3)           | ICS1, IXIARO, JESPect, or JEEV (Intercell AG)    |
| Cell culture-derived live-attenuated chimeric | SA<sub>a</sub>-14-2       | ChimeriVax-JE, IMOJEV, JE-CV, or THAUJEV (Sanofi-Aventis) |

<sup>a</sup>BIKEN, Research Foundation for Microbial Diseases of Osaka University. <sup>b</sup>CDIBP, Chengdu Institute of Biological Products.

2 mo of age and older, and the need for and timing of a booster dose to extend the duration of protection are being assessed.

A number of earlier studies have shown that the ChimeriVax-JE vaccine is safe, immunogenic, and protective in mouse and nonhuman primate models<sup>233-237</sup> as well as in small-scale human studies.37,238,239 Recently, a clinical trial in adults (aged ≥18 y) found that a single dose of IMOJEV generates a level of near-complete seroconversion (~99%) similar to that induced by 3 doses of JE-VAX (~95%), with ~94% of the participants seroconverting within 14 d.240 A 5-y follow-up study has indicated that ~87% of the 1-dose vaccinees who were seroprotected at month 6 were still protected at month 60, and this percentage increased to ~96% with a booster at month 6.241 In Thailand and the Philippines, a clinical trial in children (aged 12 to 18 or 24 mo) has shown that single-dose administration elicits a high protective immune response capable of seroconverting ~95% of naïve toddlers.242,243 There has been no indication of any serious safety concerns related to vaccination.240,241,243 Furthermore, the ChimeriVax-JE virus has been shown to be restricted in its ability to infect and replicate in six different mosquito species following oral feeding of artificial blood meals with high titers of the virus, although all the mosquitoes are susceptible to JEV infection.244,245 ChimeriVax-JE virus is thus less likely to be transmitted by mosquitoes from vaccinated persons to other hosts.

**Experimental vaccines**

The E protein of JEV has high potential for use as an immunogen capable of eliciting protective immunity, since JEV-neutralizing antibodies alone are sufficient to confer protection against infection.246,247 Ongoing efforts to develop new JE vaccines can be categorized into three different classes: (1) recombinant protein-based vaccines, (2) poxvirus-based vaccines, and (3) plasmid DNA-based vaccines.248

**Recombinant protein-based vaccines**

In *E. coli*, antigenic portions of the E protein have been expressed that generate a wide range of neutralizing antibody titers in mice.249-252 In line with this result, it is intriguing to note that a 27-amino acid peptide from the E protein, fused to Johnson grass mosaic virus coat protein to form virus-like particles, has been shown to induce JEV-neutralizing antibodies in mice and protect them against a lethal JEV challenge.253 In a baculovirus-insect cell system, the E protein has also been expressed alone or together with another viral protein, prM or NS1, that elicits neutralizing antibodies and protects mice against a challenge with JEV.254,255

Moreover, in mammalian cells, co-expression of the prM and E proteins has been shown to produce a secreted form of subviral particles capable of inducing JEV-neutralizing antibodies and generating JEV-specific cytotoxic T cells in animals.256-259

**Plasmid DNA-based vaccines**

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**Vaccine efficacy and JEV genotype replacement**

Over the past 2 decades, there has been a dramatic shift in the dominant genotype of JEV circulating in JE endemic areas.
Historically, GIII was the most widely distributed genotype since the 1990s, however, GI has replaced GIII as the major genotype emerging in many Asian countries, including China, Japan, South Korea, Taiwan, Thailand, and Vietnam. On the other hand, all currently licensed JE vaccines are derived only from GIII JEV strains, i.e., Nakayama, Beijing-1, Beijing-3, and SA14-14-2. This raises a concern that JEV strains of different genotypes exhibit antigenic differences, which may affect vaccine efficacy. In mice, a range of variations has been observed in the immunogenicity and protective efficacy of current GIII JE vaccines against heterologous JEV genotypes. In humans, a recent study has found that neutralizing antibodies, elicited by JE-VAX among Taiwanese children, show reduced neutralizing potency against emerging GI JEV strains. Similarly, decreased cross-neutralizing responses to heterologous JEV genotypes have also been observed with ChimeriVax-JE. In line with these findings, a GI JEV has been isolated from the cerebrospinal fluid (CSF) of a Chinese patient who had been vaccinated with SA14-14-2. Moreover, the reduced capacity of neutralizing antibody against different GIII JEV strains has been described in some individuals immunized with JE-VAX, SA14-14-2, and IC51. Thus, genotype replacement, in addition to the strain-specific immune response, may have important implications for future JE vaccine development.

**Recommendations for the use of JE vaccines among travelers**

From 1973 to 2008, a total of 55 JE cases were documented in travelers from non-endemic areas. In a detailed review of 37 patients, 24 (65%) appear to have spent ≥1 mo in JE-endemic areas, and most had factors that could increase the likelihood of acquiring JEV. Thus, for most travelers to Asia, the overall risk of JE is generally very low, but it varies depending on risk factors (i.e., location, season, duration, and activity), with an estimated incidence of <1 case per 1 million travelers. According to the United States Advisory Committee on Immunization Practices (ACIP), JE vaccine is recommended for travelers who spend ≥1 mo in JE-endemic areas during a high-risk period of JEV transmission. Also, the ACIP recommends that JE vaccine should be considered for individuals (1) traveling to endemic areas for a short period of time (<1 mo) during the JEV transmission season, if they visit outside of an urban area where there is an increased risk of JEV infection; (2) traveling to an area with an ongoing JE outbreak; and (3) traveling to endemic areas, with uncertainty in their destination, duration of stay, or activities. JE vaccine is not recommended for short-term travelers whose trips are restricted to urban areas or at times outside of a defined JEV transmission season.

**Immune Responses**

Both innate and adaptive immunity are important for the control of JEV infection. The innate immune response is indicated by a level of interferon-α detected in the plasma and CSF of patients with acute JE, but treatment with interferon-α-2a produces no significant improvement in the outcome of JE patients. Experimentally, a critical role for interferon-α in resolving JEV infection is suggested by the uncontrolled growth of the virus in mice lacking a functional interferon-α receptor. Of the 2 arms of adaptive immunity, the humoral immune response to JEV has been well characterized. In patients with acute JE, JEV-specific IgM and IgG antibodies are typically detectable in serum and CSF within 7 and 30 d after infection, respectively. A failure to generate an early, vigorous JEV-specific IgM and IgG antibodies in serum and CSF is associated with a higher risk of severe and fatal outcomes. In agreement with this finding, a central role of JEV-neutralizing antibodies in the protection against JEV infection has been shown in animals by administering the antibodies before or soon after infection. Thus, protection against JEV depends on virus-specific humoral immunity, and JEV-neutralizing antibodies alone are sufficient for protection.

Unlike humoral immunity, the functional significance of cellular immunity in controlling JEV infection is not well understood. In spider monkeys, which normally produce subclinical symptoms after intracerebral inoculation of JEV, flaccid paralysis develops rapidly when T-cell function is modulated by cyclophosphamide (an immunosuppressive agent). In mice, a definite role for CD4+ T cells in protection from lethal JEV infection has been demonstrated by adoptive transfer of JEV-immune cells. The same approach has suggested a possible role for CD8+ T cells in JEV clearance, since both CD4+ and CD8+ T cells are reported to be necessary for protecting adult mice against lethal intracerebral challenge with JEV. Infection experiments in a group of knockout mice have shown the relative contribution of cell-mediated immunity in protection against JEV, including (1) a critical role for CD4+ T cells in maintaining potent IgM and IgG antibody responses to JEV (using mice that lack major histocompatibility complex class II); and (2) a marginal role for CD8+ T cells in controlling JEV growth in the CNS, with indication that they are dispensable for recovery from JEV infection (using mice that lack key effector molecules [Fas receptor, perforin, or granzymes] of cytolytic CD8+ T cells). In agreement with these preclinical data, peripheral blood mononuclear cells from both JE patients and vaccinees have demonstrated JEV-specific CD4+ and CD8+ T-cell proliferation. Also, a small number of CD4+ T-cell clones that recognize the JEV E protein in an HLA-restricted manner have been established from JE vaccine recipients. Further investigation is needed to better understand the role of cellular immunity in controlling JEV infection.

**Reverse Genetics**

Reverse genetics is a powerful system for the genetic manipulation of the JEV genomic RNA that allows us to produce recombinant viruses entirely from a molecularly cloned cDNA. In 2003, we reported the first description of a reverse genetics system for JEV, developed using the highly virulent strain CNU/LP2. This system utilized a bacterial artificial chromosome (BAC) based on a single-copy fertility plasmid (F-plasmid),
which houses a genome-length cDNA of JEV (Fig. 5). Run-off transcription of the full-length cDNA in vitro generated synthetic RNAs, which were highly infectious in JEV-susceptible BHK-21 cells and had a specific infectivity of ~1 × 10^6 PFU/µg; these cells produced a high titer of synthetic viruses (~5 × 10^6 PFU/ml). Importantly, the infectious JEV cDNA was shown to be genetically stable over 180 generations of propagation in E. coli. Over the past 10 y, infectious JEV cDNA technology has contributed significantly to our progress in understanding the molecular basis of JEV replication and identifying the viral factors involved in JEV pathogenesis, including (1) the discovery of a complex RNA motif, defined by 3 discontinuous 5-nucleotide-long strands, that is a key element with a crucial regulatory role in the genome replication of JEV and other closely related flaviviruses, (2) the construction of a detailed genetic map of the 3’ cis-acting elements in JEV genomic RNA, which regulate the cell type-dependent replication of JEV and perhaps other closely related mosquito-borne flaviviruses, and (3) demonstration of the functional importance of the single highly conserved N-glycosylation motif in prM, which is crucial for multiple stages of JEV biology: prM biogenesis, virus release, and pathogenesis.

In recent years, we have also constructed another full-length infectious cDNA for JEV SA₁₄-14-2, the most widely used vaccine strain. By employing the same strategy used for JEV CNU/LP2, the genomic RNA of SA₁₄-14-2 was first cloned as 4 overlapping cDNA fragments, which were then sequentially joined at natural restriction sites present within the genome into a genome-length cDNA in the BAC plasmid pBeloBAC11 (Yun SI and Lee YM, unpublished). We have observed that molecularly cloned viruses rescued from the infectious SA₁₄-14-2 cDNA have in vitro growth properties and in vivo attenuation phenotypes identical to those of the parental uncloned SA₁₄-14-2 virus (Yun SI and Lee YM, unpublished). This system, together with the virus pair SA₁₄ and SA₁₄-14-2, now offers a unique opportunity to understand the molecular basis of how SA₁₄-14-2 virus is attenuated in virulence. Also, the infectious SA₁₄-14-2 cDNA technology has direct application to the design of a novel and promising class of vaccines to expand and improve the currently available preventive arsenals against infection with JEV and other taxonomically related flaviviruses. Furthermore, this technology will allow us to explore SA₁₄-14-2 as a vaccine vector that can express a heterologous protein and induce protective immune responses against the inserted antigen.

Conclusion

JEV is a neurological disease caused by a mosquito-borne JEV. Because of the enzootic nature of its transmission, JEV, unlike smallpox and polio, cannot be completely eliminated. Since its discovery in 1935, JEV has continued to expand its activity into new territories, although several JE vaccines have been made commercially available in different parts of the world. Concern about its spread has been highlighted by the recent emergence and spread of JEV in northern Australia, making it of significant concern for global public health. One of the most important research areas is the development of an ideal JE vaccine: one that is safer, cheaper, and more efficacious and that provides life-long protection with a single dose. The development of such a vaccine will be greatly facilitated by a deepening of our understanding of JEV replication and pathogenesis at the molecular level, which has now become technically feasible with the use of infectious JEV SA₁₄-14-2 cDNA technology. This technology also has huge potential for developing JEV SA₁₄-14-2 as a vaccine vector to deliver a foreign gene(s), as has already been achieved with infectious YFV 17D cDNA technology.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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