The National Academy of Sciences, India

Reprinted from
Proceedings of the National Academy of Sciences, India
Section of Biological Sciences
Vol. 80, Part III, 2010

5, Lajpatrai Road, Allahabad-211002, INDIA

PROCEEDINGS

OF THE

NATIONAL ACADEMY OF SCIENCES, INDIA
2010

Vol. LXXX SECTION-B PART III

Japanese encephalitis vaccines: current status and future prospects

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Received January 14, 2009; Revised December 16, 2009; Accepted January 5, 2010

Abstract

Japanese encephalitis virus (JEV) is the most important cause of viral encephalitis commonly known as "brain fever". It appears in the form of frequent epidemics throughout Southeast Asia, China, India and the Asia-Pacific belt. The disease is caused by a flavivirus named Japanese encephalitis virus that is spread to humans by the bite of infected Culicine mosquitoes, primarily *Culex tritaeniorhynchus* and *Culex vishnui*, in the Indian context. A mouse brain-derived inactivated vaccine was available until recently, but it has been discontinued largely due to safety concerns. A cell culture-derived attenuated JE vaccine is produced in China and consumed in several Asian countries, including India, but this is not manufactured as per internationally acceptable norms. Several new promising JE vaccine candidates are under various stages of development, some of which have entered clinical trials. These new candidate JE vaccines are cost-effective and have the potential to generate long-lasting immunity.

Keywords: JEV, VLP, recombinant virus, immunogenicity, vaccine

सारांश

विषाणु मस्तिष्कशोथ का, जिसे सामान्य रूप में "मस्तिष्क ज्वर" कहत हैं, जापानी एनसिफैलाइटिस विषाणु (जेईवी) सबसे महत्वपूर्ण कारक है। संपूर्ण दक्षिणपूर्वी ऐशिया, चीन, भारत और एशिया-पेसिफिक क्षेत्र में यह अक्सर होने वाली महामारी के रूप में प्रकट होता है। यह रोग एक फ्लेवीविषाणु द्वारा होता है जिसे जापानी एन्सिफेलाईटिस विषाणु कहते है, जो भारतीय परिवेश में मानव जाति में, संक्रमित क्यूलीसीन मच्छरों, प्रमुखतः *क्यूलेक्स ट्राईटिनियोरिंकस* और *क्यूलेक्स विश्नुई* के काटने से होता है। हाल के समय तक, चूहे के मस्तिष्क से प्राप्त निष्क्रिय टीका उपलब्ध था परंतु सुरक्षाकारणों से इसका प्रयोग बंद कर दिया गया। चीन में बनाया गया, कोषिका संवर्धन से प्राप्त क्षीण किया हुआ जे.ई. टीके का, भारत सहित अनेक एशियाई देशों में उपभोग किया जाता रहा है परंतु इसका निर्माण अंतराष्ट्रीय स्वीकार्य मानकों के अनुसार नहीं किया जाता है। अनेक नये वाग्दत्त टीका प्रत्याशी विकास की विभिन्न अवस्थाओं में हैं जिनमें से कुछ एक नैदानिक परीक्षणों की स्थिति में प्रवेश कर गये हैं। इन नये जे.ई. टीका प्रत्याशियों में, जो सस्ते भी हैं, लम्बे समय तक की प्रतिरोधकता पैदा करने की समर्थता है।

सांकेतिक शब्द : जेईवी, वीएलपी, पुनर्योजनी विषाणु, प्रतिरक्षाजनत्व, टीका

Introduction

Japanese encephalitis (JE) was first reported from Japan in the late 1800's, but has since spread to other parts of Asia (BOX-1, Fig. 1). It is an arthropod-borne viral disease and is the most common viral encephalitis in Southeast Asia with more than 3 billion inhabitants. The virus, Japanese encephalitis virus (JEV) is spread by the bite of *Culex* mosquitoes, predominantly *Culex tritaeniorhynchus* and *Culex vishnui*, in the Indian context. The major amplifying vertebrate hosts are domestic pigs. Wading and migratory birds are also involved in the transmission cycle. Man is an incidental host and viral titres are usually very low, so that further transmission does not occur. For this reason, humans are regarded as dead-end hosts (Fig. 2).

Box-1

Japanese Encephalitis - Historical Facts

- 1870's - First JE outbreak in Japan
- 1924 - Isolation of JEV from a fatal case of encephalitis in monkeys
- 1935 - Isolation of Nakayama strain from a fatal human case
- 1950 - Elucidation of route of virus transmission
- 1955 - First Indian JE cases in Vellore, Tamil Nadu
- 1973 - First Indian JE epidemic in Bankura, West Bengal
- 1978 - Gorakhpur strain (GP78) was isolated
- 1990 - JE spreads to Western and North Western India
- 2006 - India imported SA 14-14-2 vaccine from China

Box-2

Japanese Encephalitis fact sheet

Geographic range	India, Pakistan, Nepal, China, South Asia, Southeast Asia, Oceania, Northern Australia
Major vectors	*Culex tritaeniorhynchus, Cx. vishnui, Cx. pipiens, Cx. gelidus, Cx. fuscocephala*
Major vertebrate hosts	Domestic pigs, migratory birds, ardeid (wading) birds
High risk groups	Infants and children below 10 years, elderly, non-immune adults, immunocompromised individuals
Annual incidence of disease	~50,000
Annual mortality	~10,000
Annual morbidity	~15,000

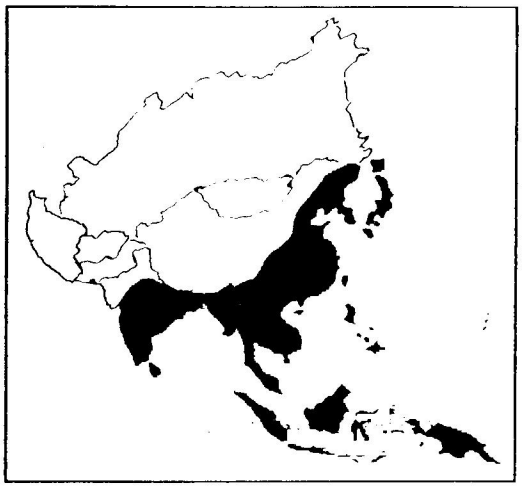

Fig. 1– Geographic distribution of Japanese encephalitis. Source : Centers for Disease Control and Prevention (CDC), Atlanta

Following a bite from an infected mosquito, the virus amplifies and disseminates through the body by the blood-vascular system. JEV, being neurotropic, eventually enters the central nervous system (CNS), predominantly colonizing the thalamus. Symptoms of febrile illness develop after 5-15 days of incubation. In cases of severe disease, patients display neurological symptoms, including behavioural changes, focal neurologic deficits, generalized hypotonia, movement disorders, often resembling Parkinsonism, accompanied by seizures. The cardinal clinical feature of this disease is encephalitis. The annual incidence of disease is ~50,000, with mortality amounting to ~10,000. The disease predominantly affects infants and young children and the survivors are left with life-long neuro-psychiatric sequelae, a major complication of JE (BOX-2).

The majority of adults living in endemic areas have acquired immunity against JEV. However, travellers to endemic areas are particularly at risk, depending on the season of their visit and the nature of their outdoor activities, inspite of the fact that only 3% of the vector mosquitoes are infected, even in hyperendemic areas[1]. There is no specific treatment for JE. Only supportive care can be provided to alleviate the symptoms. JE cannot be totally eradicated due to its animal reservoir, but the disease can be held at bay by effective vaccination. However, the only licensed JE vaccine, the mouse brain-derived JE vaccine has been withdrawn from the international market due to inherent drawbacks pertaining primarily to safety. Moreover, Central Research Institute (CRI), Kasauli, the only JE vaccine manufacturer in India has recently stopped its production as its vaccine production process was not compliant with the current international norms. Thus, there is a pressing need to develop a safe and effective JE vaccine. Efforts in this direction are going on, both in India and abroad, and several promising vaccine candidates are currently in various stages of development.

Epidemiology

Two major epidemiological patterns of disease are observed. These are either endemic cases or epidemic cases. These two patterns are confined to specific geographical areas. In northern areas, such as northern Vietnam, northern Thailand, Korea, Japan, Taiwan, China, Nepal, and northern India JE occurs in the form of epidemics during the summer/monsoon months. The southern areas, such as southern Vietnam, southern Thailand, Indonesia, Malaysia, Philippines, Sri Lanka and southern India are endemic for

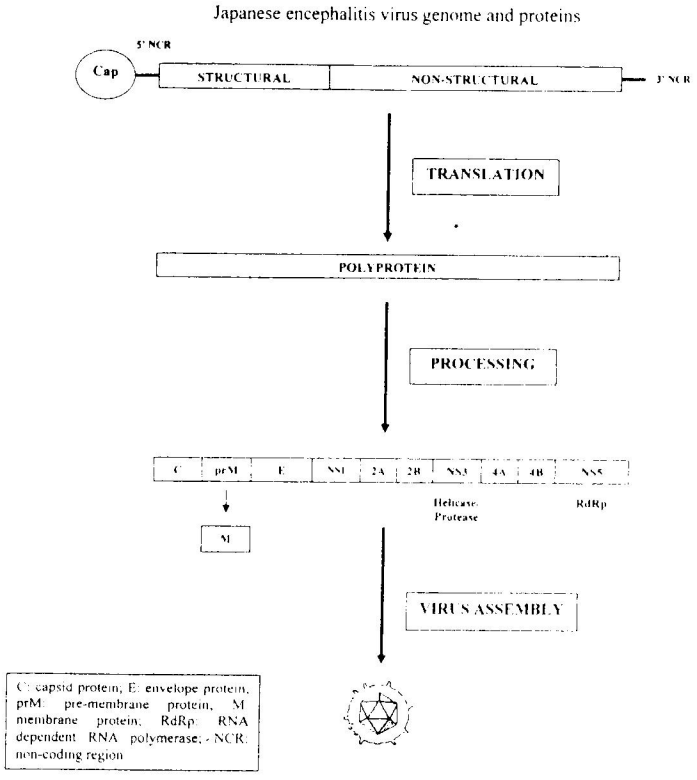

Fig. 2

JE and cases occur sporadically throughout the year with a peak after the onset of the monsoon season. The Indian scenario reveals that JE is progressively moving westwards, and it would not be very surprising if it soon spreads throughout India.

Pathogenesis

JEV, after being injected by the bite of infected Culicine mosquitoes, penetrates the skin, and the virus multiplies in the Langerhans-type dendritic cells in regional lymph nodes. After a transient phase of viremia, invasion of the CNS takes place by the haematophagus way or via the endothelial system. Unknown factors allow the breakdown of the blood-brain-barrier (BBB), but it could be facilitated by neurotransmitters. CNS lesions are predominantly seen in the thalamus and the cerebral peduncles; however, lesions can also be found in the substantia nigra, the cerebral and the cerebellar cortex as well as in the anterior horn cells of the spinal cord. JEV has also been reported to infect the developing fetus transplacentally and cause abortions[2].

The pathogen

JEV, the aetiologic agent of JE, belongs to the family Flaviviridae, which contains many other flaviviruses of immense medical importance (Table 1). JEV is a single-stranded, positive-sense RNA virus. It's approximately 11 kB genome consists of a single open reading frame (ORF) that encodes a single polyprotein of approximately 3,400 amino acids[3,4]. This polyprotein is subsequently cleaved co- and post-translationally by host and viral proteases into three structural and seven non-structural proteins. The three structural proteins include envelope (E), membrane (M) and capsid (C). The non-structural proteins include NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (FIGURE 2). Of all these proteins, the viral envelope (E) protein is of considerable importance from a vaccine development standpoint. The reason for this being the fact that all the neutralizing (Nt) epitopes are present on the surface of the E protein and it is the Nt antibodies that are the proven primary mediators of protective immunity against JEV infection. It follows that the majority of the vaccine development efforts concentrate on the E protein as the vaccine antigen, largely due to the reasons highlighted above.

Prevention

No specific treatment is currently available for the management of JE patients. Moreover, since JE is a zoonosis, it can never be totally eradicated from the

face of the earth by measures such as vaccination. However, efficient and sustained vaccination campaigns can most certainly keep the disease at bay. Vector control measures, though having certain limitations, both economically and logistically, should be in place to buttress the vaccination efforts. The various vaccination strategies currently in development have been discussed in the following sections in details as this is the most feasible preventive measure available to control JE.

Table 1 Major human flaviviral diseases and their endemic areas.

Disease	Endemic areas
Japanese encephalitis	India, China, South-east Asia and Oceania
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

Justification for vaccination

There is no effective drug treatment for the disease. Moreover, there is no effective method of environmental control of JE transmission. Although improvements in agricultural practices due to betterment of socio-economic status have led to reduced disease burden in some countries, large-scale vaccination of susceptible human populations appears to be the logical approach towards controlling JE, both now, and in the future[5]. The effect of mass-scale vaccination against JE has already shown great promise in various regions of China and has led to decreased disease burden in countries like Japan, Korea and Thailand, hence justifying vaccination efforts.

Who should be vaccinated?

Japanese encephalitis vaccine is recommended for native and expatriate residents of endemic areas, laboratory workers potentially exposed to the virus, and for travellers spending 30 days or more in endemic areas. It should be borne in mind that vaccination against JE in endemic areas will have to be a concerted effort as approximately 3 billion people currently live in JE-endemic regions, where more than 70 million children are born each year. It should be noted that given the mostly infrequent occurrence of JE in early infancy and the likely interference with passively acquired maternal antibodies during the first months of life, vaccination is not recommended for children below the age of 6 months.

Current vaccines against JE

The only JE vaccines that are currently available are manufactured in China and are based on the mammalian cell-culture system. These vaccines are either inactivated or live-attenuated. The inactivated vaccine utilizes the Beijing P-3 strain of JEV that has been propagated in Primary Hamster Kidney (PHK) cells. This inactivated JE vaccine has now largely been replaced by the live attenuated vaccine. This vaccine utilizes the stably neuro-attenuated SA 14-14-2 strain of JEV, but continues to propagate the virus in the PHK cell line. The PHK cell line is not a WHO-certified vaccine substrate, a major hindrance for the vaccine's large-scale acceptability, in spite of its high efficacy and safety. This vaccine is manufactured by the Chengdu Institute of Biological Products (CDIBP), China. The vaccine has been used for over 20 years in over 200 million children in China since its licensure in 1988[6]. Other countries that have licensed and used the vaccine are South Korea (since 2001), Nepal (since 1999), and India (since 2006). In India, vaccination with this vaccine started on 15th May, 2006, with almost 10 million children in 4 states and 11 districts being vaccinated. With regard to the 65 serious adverse events (including 22 deaths) reported after mass vaccination campaigns in India, an expert committee constituted by the WHO confirmed that these were not related to the imported SA 14-14-2 vaccine[7]. A recent study in the Philippines has revealed that the SA 14-14-2 vaccine can be safely administered along with measles vaccine in 9 month old infants without any loss of immunogenicity in either of the vaccines[8]. Combined administration along with other vaccines currently used in the national immunization program need to be carried out so that SA 14-14-2 vaccine can, in the future, be incorporated into the routine childhood immunization program. A major advantage of this vaccine is that it is inexpensive and hence would be affordable by the economically weak target population.

Until a few of years back, the mouse brain-derived inactivated JEV vaccine (JE-VAX®), manufactured by BIKEN (Japan) and various other companies around the globe, such as CRI (Kasauli, India), was the only licensed JE vaccine that was available internationally. However, due to certain inherent drawbacks, pertaining particularly to safety[9], the vaccine has been withdrawn from the international market. JE vaccine manufacture in India has also ceased[10]

and hence, there is great urgency to develop a cost-effective JE vaccine.

JE vaccines under development

Quite a few innovative JE vaccines are currently under various stages of development. These include vaccines based on recombinant proteins, virus like particles (VLPs), infectious clones, or simply "naked DNA". These approaches encompass various vaccine technologies, including prokaryotic and eukaryotic expression systems, mammalian cell culture approaches, development of chimeras involving the yellow fever virus 17D as a vaccine construct backbone or the development of plasmid DNA constructs. The new generation vaccines should ideally stimulate both the cellular and humoral arms of the adaptive immune system for effective protection against JEV infection. The recent developments in this field are summarized and evaluated as under.

Vero cell culture-derived vaccines

Various laboratories have focused their attention on Vero cells, derived from the African green monkey *(Cercopithicus aethiops)* kidney to propagate the vaccine virus. For more than a decade, Vero cells have been used to manufacture polio as well as rabies vaccines with excellent records of safety, thus advocating Vero as a safe cell-line for producing vaccines for human use [11].

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-inactivated vaccine (PIV) was made which induced high titres of JEV Nt antibodies in mice in a dose-dependent manner after two injections [12]. The vaccine protected mice against morbidity and mortality after challenge with virulent JEV. Compared to the mouse brain-derived vaccine, the Vero cell-derived JE-PIV was more immunogenic and as effective at preventing encephalitis in mice.

In Japan, using the Beijing-1 strain of JEV grown in Vero cells, an industrial-scale process conforming to cGMP criteria has been developed [13]. In pre-clinical and small-scale phase I clinical trials, the vaccine was found to be safe and its effectiveness to be equivalent to that of the mouse brain-derived inactivated vaccine [14]. However, clinical trials using a larger sample size are required to further validate the safety and effectiveness of this vaccine.

Intercell (Austria) has developed a Vero cell-derived JE vaccine IXIARO® that utilizes the SA 14-14-2 neuro-attenuated strain of JEV. This vaccine candidate uses a two dose vaccination regimen and has successfully undergone pivotal phase III clinical trials[15]. This vaccine has been approved by the US FDA on March 30, 2009 and has also received marketing authorization by the European Commission on April 2, 2009.

The authors have developed a Vero cell-derived formaldehyde-inactivated JE vaccine using P20778, an Indian strain of JEV[16]. These studies indicated that virus inactivation with formalin at 22°C, which required shorter incubation period, was as good or better than virus inactivation at 4°C as 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 following cGMP guidelines.

Recombinant protein vaccines

The E protein of JEV and other flaviviruses are involved in such important functions as receptor binding and membrane fusion and have been shown to induce virus-neutralizing antibodies. As the virus Nt antibodies are considered as the cardinal mediators 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 murine model, the details of which are presented below.

The Escherichia coli system

One of the earliest studies using the *E. coli* platform demonstrated that 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[17]. 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[18]. Although the immunized mice were protected against lethal JEV challenge, utility of this vaccinogen comprising a single epitope is very limited.

The authors have evaluated the possibility of developing a peptide vaccine against the domain III

fragment of JEV E protein (E-DIII). Two constructs were tested, one bearing an MBP-tag, while the other a His-tag. Immunization studies were carried out in mice using Freund's adjuvant or alhydrogel. In both cases, Nt antibodies were generated that protected the mice against a lethal dose of JEV. The antibody-inductive as well as protective efficacy of the E-DIII-His in combination with alhydrogel proved to be superior, and comparable to the commercial mouse brain-derived vaccine[19].

The authors have also evaluated the possibility of developing an oral vaccine against JEV[20]. 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 made high titers of anti-E and anti- 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.

The baculovirus 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 [21]. 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-neutralizing antibodies, although the titres 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[22], baculovirus recombinants were constructed that synthesized the E protein or the NS1 glycoprotein of JEV individually or together. Around 70% protection was recorded in mice immunized with cells infected with recombinant virus synthesizing the E or the E+NS1 proteins, compared to 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 sub-optimal, since only 70% of the control mice succumbed to the challenge. However, in a subsequent study [23] the baculovirus-expressed JEV E protein was found to be more efficacious. 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% 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.

Cell culture system

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 sub-viral particles (SVPs) that were highly immunogenic in mice inducing JEV Nt antibodies and virus-specific memory T-lymphocytes that conferred protection against live virus challenge[24, 25]. Chinese hamster ovary (CHO), COS-1 and rabbit kidney-derived RK13 cells, permanently transfected with the JEV prM-E sequence showed continuous production of extracellular particles (EPs) which generated Nt antibodies in immunized mice that protected them against lethal JEV challenge [26, 27]. 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 vaccines

Recombinant viruses are useful tools in vaccine research. A variety of viruses have been investigated as potential vectors for developing recombinant viruses. An important feature of almost all recombinant viruses is their ability to induce both humoral as well as cell-mediated immune responses. Canary-pox, vaccinia, yellow fever, and more recently, adenoviruses have been used for the development of recombinant vaccines against JE. These are briefly reviewed as under.

Recombinant poxvirus system

Konishi *et al*[28] constructed vaccinia recombinants expressing different JEV proteins. They showed that vaccinia recombinants co-expressing 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, although generated anti-NS1 antibodies, these induced only a low level of protection in mice against lethal JEV challenge.

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 [29]. 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 [30]. The immunized pigs developed lower levels of viremia compared to the non-immunized controls, when challenged with 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 (MVA) strain. 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[31].

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. Canarypoxvirus has received the most attention as the recombinant vaccine vector since it induced immunity more efficiently than other avipoxviruses. ALVAC is an attenuated vaccine strain of canarypoxvirus. Mice immunized with an ALVAC recombinant expressing the prM, E and the NS1 proteins of JEV produced JEV Nt antibodies that protected them from a lethal JEV challenge[32].

Raengsakulrach *et al*.[33] have evaluated NYVAC-JEV and ALVAC-JEV recombinants in rhesus monkeys where they were found to be safe and immunogenic to varying degrees. These recombinants were further evaluated in a controlled, randomized, double-blind clinical trial to assess safety and immunogenicity in human volunteers [34]. 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 candidate induced Nt antibodies only in the vaccinia-naïve 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 system

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. During the 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[35]. Recombinant adenovirus, RAdEs, synthesizing the carboxy-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 intra-muscularly (IM), generated high titres of JEV Nt antibodies and splenocytes from these animals secreted large amounts of IFN-γ in the presence of JEV and showed cytotoxic activity against JEV-infected cells. Naïve mice immunized with RAdEs were completely protected against a lethal intra-cerebral (IC) challenge with JEV. Mice with pre-existing Ad5 Nt antibody titres similar to those in young children, could also be immunized successfully by RAdEs demonstrating its potential as a candidate vaccine for children, the primary targets of JEV infection.

Recombinant yellow fever virus system

Yellow fever (YF) 17D is a live, attenuated vaccine that has been in use for nearly 70 years with 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 vaccines. 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.

Chambers *et al*[36] constructed a chimeric virus (ChimeriVax-JE) 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. Following successful pre-clinical development in murine and primate models, ChimeriVax- JE en-

tered a randomized, double-blind clinical trial which demonstrated that the vaccine candidate induced JEV Nt antibodies in 100% of naïve (n=6) as well as YF17D-immune (n=6) subjects[37]. JE antibody levels were higher in YF-immune than in naïve subjects, dispelling concerns about the anti-vector 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[38]. Significantly, immunological memory was demonstrated in ChimeriVax-JE immunized individuals in the form of an anamnestic response following the challenge with the mouse brain-derived inactivated JE-VAX®.

DNA 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). 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 does not pose 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 decade, quite a few candidate DNA vaccines for JE have been developed and tested in animal models with varying degree of success. These are reviewed as under.

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 E protein in the correct conformation. As a result, the majority of JEV DNA vaccine constructs incorporate both E and prM.

Although early studies with JEV DNA vaccine candidates encoding prM and E genes elicited low titres of Nt antibody in mice, it was subsequently demonstrated that a single IM injection of as little as 25 μg of recombinant plasmid DNA, encoding the JEV prM and E proteins, could induce JEV Nt antibodies capable of protecting the immunized mice against lethal viral challenge[39]. This remarkable finding stems 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[40] have evaluated the immunogenicity of a plasmid that contained the JEV E-encoding sequence without any signal peptide. 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.

Considering that the form of the immunogen might affect its immunogenicity, the authors studied the immunogenicity in mice of plasmidos, delivered IM or ID (using a gene gun) that synthesized the membrane-anchored (pMEa) and the secreted (pMEs) forms of the JEV E protein [41]. Form of the E protein or the route of DNA delivery did not affect the level of protection seen in immunized mice where about 60% protection was seen in an IC JEV challenge model. This was significantly lower than ~90% protection afforded by the commercial formalin-inactivated vaccine in mice. The plasmid constructs were recently evaluated in a primate *(Macaca mulatta)* model, where the DNA vaccine candidates efficiently primed the immune system that subsequently generated a vigorous anamnestic immune response upon a sham challenge with the mouse brain-derived inactivated vaccine, used as a control[42].

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. Firstly, by investigating the delivery of plasmid DNA in a particulate form to enhance its uptake by the professional APCs. They found that DNA adsorbed onto cationic microparticles showed enhanced immunogenicity in mice[43]. They believe that use of smaller cationic particles in the nano-range would further enhance the efficacy of the candidate DNA vaccine. Secondly, by investigating the co-administration of JEV DNA vaccine and plasmids encoding the cytokines interleukin-2 (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 co-administration of JEV DNA vaccine and plasmid encoding GM-CSF using the gene gun enhanced anti- JEV antibody titres resulting in an increased level of protection in mice against lethal JEV challenge [44]. At present, work is in progress to enhance

Table 2– Vaccines against Japanese encephalitis

Vaccine	Type	Virus used	Manufacturer	Dosing	Status
BIKEN/JE-VAX®	Mouse brain-derived; Inactivated	Nakayama	BIKEN, Japan; Sanofi Pasteur, USA, Green Cross, South Korea; CRI, India	Three doses at 0, 7, 30 days. Boosting after 1 year and subsequently, every 3 years till child is 10 years of age.	Withdrawn from international market; Manufacture stopped at CRI
SA 14-14-2	PHK cell-cultured; Live-attenuated	SA 14-14-2	Chengdu Institute of Biological Products, China	Single dose	Currently in use in China, India, Korea, Sri Lanka and Nepal
IXIARO®	Vero cell- cultured; Inactivated	SA 14-14-2	Intercell, Austria; Indian partner: Biological E	Two doses at 0 and 30 days	Pivotal Phase III trials completed; US FDA approved in March 2009; European Commission granted, Marketing authorization in April 2009
Chimeri-Vax-JE™	Infectious clone; Live-attenuated	JEV SA 14-14-2 and YF 17D chimeric infectious clone	Acambis, UK	Single dose	Phase III trial in USA, Australia and Asia

the immunogenicity of the DNA vaccine candidate by making fusion constructs that would target the antigen to particular cellular compartments or target certain receptors on the surface of APC's for efficient antigen delivery.

Conclusions

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. Though there is some hope that antiviral agents may be found in near future, for the present, vaccination appears to be the only logical alternative. However, it should be borne in mind that JE cannot be totally eradicated as for diseases like smallpox, polio or measles, where humans are the only host. At the most, this zoonotic disease can be held at bay by the judicious vaccination of humans.

Now that the manufacture of the mouse brain-derived inactivated vaccine has been discontinued, there is all the more urgency to develop a safe and effective vaccine against JE. 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 the cost. Policy makers and health officials should bear in mind that the target populations for the JE vaccines are the economically weaker strata of the society. This is an important reason why the mouse brain-derived vaccine could not be used for mass-scale vaccination.

At present there are quite a few promising JE vaccine candidates that are in various stages of development (Table- 2). Vero cell-derived inactivated JEV vaccine and YF virus based recombinant vaccine ChimeriVax-JE, are under advanced clinical evaluations. JEV DNA vaccine candidates have been evaluated in non-human primates[42, 45] and may enter clinical trials soon. Moreover, Intercell's IXIARO® has been approved by the US FDA and received the go ahead by the European Commission last year. The coming five 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 long lasting immune response. Besides, vaccines that could be administered by non-invasive means would be most desirable.

Acknowledgments

The work is supported by grants from the Department of Biotechnology, Govt. of India and from

the core grant of the National Institute of Immunology, New Delhi.

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