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Review

Development of a recombinant vaccine against Japanese encephalitis

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Japanese encephalitis (JE) is the major form of viral encephalitis in much of the South-East Asia, India, and China. The disease is caused by a mosquito-borne virus known as Japanese encephalitis virus (JEV). The virus spreads in the form of epidemics, although several endemic areas for JEV activity are known. In recent years, JEV has spread to newer geographic locations such as Australia and Pakistan, and thus has become an important emerging virus infection in these areas. A mouse brain–derived, formalin-inactivated vaccine is available for immunization against JE. Because the formalin-inactivated JEV vaccine has limitations in terms of safety, availability, and cost, attempts are being made to develop improved vaccine using the recombinant DNA technology. This article reviews various attempts in this direction and summarizes the latest developments such as the recombinant yellow fever virus– or the plasmid DNA–based JEV vaccine. *Journal of NeuroVirology* (2003) **9,** 421–431.

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Japanese encephalitis (JE) is the most important form of viral encephalitis in Asia. It is caused by Japanese encephalitis virus (JEV), a member of the Flaviviridae family of animal viruses. The virus is transmitted to human beings by mosquitoes feeding on viremic animals, mostly domestic pigs, resulting in frequent epidemics of JE. The virus is active over a vast geographic area covering India, China, and virtually all of the South-East Asia. Approximately 3 billion people live in the JEV endemic areas where at least 50,000 cases of JE are reported every year. Of these, about 10,000 cases result in deaths and a high proportion of survivors develop serious neurological and psychiatric sequel (World Health Organization [WHO], 1998). In recent years, outbreaks of JE have occurred in several previously nonendemic areas and the virus has been isolated from newer geographical locations such as Pakistan (Igarashi *et al*, 1994) and Australia (Hanna *et al*, 1996, 1999; van den Hurk *et al*, 2001). The high fatality rate and the frequent residual neuropsychiatric complications in survivors make JE a considerable health problem in many Asian regions and it has become an important emerging infection in Australia (Mackenzie *et al*, 2002).

JEV contains a plus-sense, single-stranded RNA genome of ∼11 kilobases (Sumiyoshi *et al*, 1987; Vrati *et al*, 1999). The genomic RNA contains a single open reading frame capable of encoding a polyprotein of ∼3400 amino acids that is subsequently cleaved, co- and post-translationally, by both host and viral proteases, into several structural and nonstructural viral proteins. A lot of information about JEV proteins is derived from studies on other flaviviruses and is assumed to apply to JEV because of the high level of similarity of its genome structure and organization to other flaviviruses (Chambers *et al*, 1990; Westaway *et al*, 2002). Thus, the JEV polyprotein is cleaved to produce three structural (capsid, C; membrane, M; and envelope, E), and seven nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins. The C protein (12 to 14 kDa) is positively charged and forms the structural component of the virus nucleocapsid. The M protein (8 to 9 kDa) is formed by

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cleavage and removal of the N-terminal segment from its precursor, the premembrane protein (prM), which is 18 to 19 kDa in size. The cleavage is caused by a process presumed to be linked to the maturation of the envelope glycoprotein and the development of virus infectivity (Guirakhoo *et al*, 1992). However, in certain instances, prM cleavage may not be complete, thus allowing the prM protein to be an additional target on virions for neutralizing antibodies (Bray and Lai, 1991). The E glycoprotein (53 to 55 kDa) is an outer structural protein of the virus that is a typical membrane glycoprotein, with a C-terminal domain spanning the membrane. It is the major virion antigen responsible for a number of important processes that include virion assembly, receptor binding, and membrane fusion. The E protein is the principal target for neutralization *in vitro* and *in vivo* by specific antibodies (Gould *et al*, 1986; Hawkes *et al*, 1988; Kimura-Kiroda and Yasui, 1988). The nonstructural protein NS1 (39 to 41 kDa) is a glycosylated protein, which is derived from the polyprotein by an N-terminal cleavage involving a novel host protease (Chambers *et al*, 1990). NS1 is believed to be involved in the assembly and release of virions (Lee *et al*, 1989). This protein is found on the cell surface and in the culture medium of infected cells (Cardiff and Lund, 1975). During the course of infection, NS1 protein evokes a strong antibody response that protects the host against challenge with flavivirus, presumably through a complement-mediated pathway (Schlensinger *et al*, 1987). In addition, mechanisms such as antibody-dependent cell cyotoxicity may also be responsible for the NS1-mediated protection (Schlensinger *et al*, 1993). NS2A and NS2B are low-molecular-weight proteins that are thought to be involved in the processing of other viral proteins (Nestorowicz *et al*, 1994). NS3 protein (68 to 70 kDa) is conserved among flaviviruses and has protease and nucleotide triphosphatase/helicase activities (Wengler and Wengler, 1989; Murthy *et al*, 2000; Bartelma and Padmanabhan, 2002). NS4A and NS4B are small proteins whose functions are not clear, although they may be involved in the membrane localization of NS3 and NS5 through protein-protein interactions (Chambers *et al*, 1990), or in the formation of the genomic RNA replication complex (Westaway *et al*, 2002). NS5 protein (103 to 104 kDa) is the largest and most conserved protein and is considered to be the viral RNA polymerase (Mandle *et al*, 1989).

There is no drug treatment for JE. Although improvements in agricultural practices have contributed to the reduction in disease incidence in some countries, JE vaccination is the single most important control measure. There is only one WHO-approved JEV vaccine that is currently available in the international market. The vaccine is developed by formalin-inactivation of JEV cultured in mouse brain. The major problems associated with the mouse brain–derived inactivated vaccine are the lack of long-term immunity and practical difficulties in production of large amounts of vaccine needed for mass immunization programs (Ku *et al*, 1994). To obtain effective protection, multiple boosts of the inactivated vaccine are routinely required, making the vaccination program costly. In addition, repeated immunizations with killed vaccine prepared from mouse brain may cause hypersensitivity reactions in vaccinees (Nothdurft *et al*, 1996).

A live, attenuated JEV vaccine based on the SA14-14-2 strain is licensed for use in China. More than 200 million doses of this vaccine have been delivered since 1988 with excellent record of safety and efficacy (Xin *et al*, 1988; Liu *et al*, 1997; Bista *et al*, 2001). However, this vaccine is produced in primary hamster kidney cells that are not approved by WHO for human vaccine production.

In the past few years, there has been a rapid growth in the knowledge of molecular information on JEV, and on the basis of this information, several potential recombinant vaccines have been developed using different approaches. This review attempts to summarize the recent developments in this field and evaluate the different systems and their potential to provide an ideal JEV vaccine that may be cheap, safe, and capable of providing long-lasting protective immunity.

The attempts to produce a recombinant vaccine against JE can be broadly divided into three categories depending on the approach used in the vaccine development. These are listed below.

Recombinant protein–based vaccines

The E protein of flaviviruses has been shown to induce virus-neutralizing antibodies besides being involved in such important functions as receptor binding and membrane fusion. E protein of JEV, therefore, has the potential to be used as the 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 JEV E protein has been tested in mice, the details of which are given below.

Envelope protein synthesized in Escherichia coli

In order to localize the neutralization antigenic site in the linear amino acid sequence of JEV E protein, Mason *et al* (1989) synthesized the small fragments of the E protein as trpE fusion proteins in *E. coli*. They found that E protein segment containing residues 303 to 396 was the shortest sequence capable of reacting with various JEV-neutralizing monoclonal antibodies. An intramolecular disulphide cross-linkage between cysteine-304 and cysteine-335 was necessary for the binding of these monoclonal antibodies. Thus the 95–amino acid antigenic domain appeared to be capable of forming several conformational neutralizing epitopes. However, this did not prove to

be an effective immunogen for inducing neutralizing or protective antibodies in mice.

Subsequently, Seif *et al* (1995), using a similar strategy of expressing the parts of the E protein in *E. coli*, found that the fragment B3, encoding a 27-residue-long sequence between amino acids 373 and 399, fused to protein A elicited the highest neutralizing antibody titer. To exclude the possible influence of the protein A contained in the fusion protein on the mouse immune response, the fragment B3 was synthesized fused with glutathione-*S*-transferase (Seif *et al*, 1996). Mice immunized with recombinant glutathione-*S*-transferase–B3 fusion protein induced an immune response (mean enzyme-linked immunosorbent assay [ELISA] titer 3364, neutralization titer 1:75) almost similar to that obtained by the recombinant protein A–B3 fusion protein (mean ELISA titer 3476, neutralization titer 1:75). Seif and coworkers, thus, concluded that the 27–amino acid sequence contained in the fragment B3 could be a suitable candidate for a recombinant JE vaccine. These workers, however, did not report on the protective efficacy of the B3 fragment. Most epitopes in an antigen are conformational and thus chances of finding a linear sequence that may provide significant degree of protection are limited. However, with the recent availability of the three-dimentional structure of JEV E protein (Kolaskar and Kulkarni-Kale, 1999), it may be possible to identify antigenic peptides capable of inducing the desired protective efficacy. In this connection, it is interesting to note that a small peptide derived from JEV E protein, fused to Johnson grass mosaic virus coat protein to form recombinant virus-like particles, induced neutralizing antibodies in mice and protected them against lethal dose of JEV (Saini and Vrati, 2003).

JEV proteins synthesized by recombinant baculovirus

The baculovirus expression system that utilizes *Autographa californica* nuclear polyhedrosis virus (AcNPV) has been used to obtain high-level expression of many foreign genes, including those that require proteolytic processing, glycosylation, or secretion (Luckow and Summers, 1988). One of the major advantages of this virus expression vector system over other systems is the abundant expression of recombinant proteins, which are, in many cases, antigenically, immunogenically, and functionally similar to their native counterparts. In addition, baculoviruses are not pathogenic to vertebrates and do not employ transformed cells or transforming elements as do mammalian expression systems. Matsuura *et al* (1989) constructed 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. Infection of *Spodoptera frugiperda* cells with the recombinant virus produced the prM and the E proteins. The E protein synthesized by the baculovirus recombinants was glycosylated and similar in size to the authentic viral protein, and was located on the surface of the infected cells. Monoclonal antibodies to JEV E protein reacted with the recombinant E protein expressed by the baculovirus. A second baculovirus recombinant was made that contained the complete coding sequence for the E protein, together with the partial sequence encoding the prM and the NS1 proteins. This recombinant synthesized the E protein that was similar in size to the native JEV E protein. However, E protein synthesized by the second recombinant failed to react to a flavivirus–cross-reactive monoclonal antibody, presumably due to altered folding of the E protein in the absence of prM protein. Mice immunized with cells infected with the recombinant viruses developed JEV-neutralizing antibodies, although the titers were lower than those seen in JEV-infected mice. Protective efficacy of the recombinant baculovirus–produced E protein was not reported.

In another study, McCown *et al* (1990) constructed baculovirus recombinants synthesizing the E or the NS1 glycoproteins of JEV individually or together. Mice were immunized with the recombinant virus–infected *S. frugiperda* cells. Importantly, around 70% protection was recorded in mice immunized with cells infected with recombinant virus synthesizing the E or the E plus NS1 proteins, compared to the 30% protection seen in the unimmunized mice. No protection was offered by immunization with the cells infected with the NS1-expressing recombinant. Neutralizing antibody was detected only in E glycoprotein recipient mice. These authors concluded that the enhanced protection seen in the immunized mice was statistically significant. It would, however, appear that the virus dose used for the challenge was low, as only 70% of the unimmunized control mice succumbed to the challenge; and thus efficacy of baculovirus-expressed JEV E protein for immunization remains to be conclusively demonstrated.

Purified subviral particles synthesized by mammalian cells

Studies described above indicated that correctly processed, glycosylated, and properly folded JEV E protein had the potential to induce neutralizing antibodies and offer protection against the live virus challenge. JEV proteins expressed in mammalian cells would be synthesized in a manner similar to natural viral infection. Konishi and coworkers (1992) have been studying the potential of JEV E protein synthesized in mammalian cells. In their initial studies, they found that HeLa cells infected with a recombinant vaccinia virus encoding the prM and E genes of JEV synthesized extracellular subviral particles (EPs). These particles contained the JEV prM/M and E proteins embedded in a lipid bilayer without any nucleic acid being associated with them (Konishi *et al*, 1992). The EPs were uniformly spherical, with a 20-nm

diameter and had 5-nm projections on their surface. Significantly, mice immunized with a single inoculation of the purified EPs emulsified with Freund's adjuvant were fully protected against a lethal viral challenge. Subsequently, Konishi *et al* (1997) studied the immunization potential of the EPs without the use of an adjuvant. They found that mice immunization with EPs containing as low as 1 μg of the E protein produced virus-neutralizing antibodies and generated virus-specific cytotoxic T lymphocytes (CTLs).

Because EPs from vaccinia-infected cells carry the risk of containing the vaccinia virus, a continuously expressing eukaryotic cell line would be an ideal way to produce EPs in terms of improved safety and higher yields. However, toxic effects due to the fusion of the cells by EPs (containing the fusion competent M/E oligomer) prevented the establishment of a cell line continuously producing JEV EPs. Thus, Konishi *et al* (2001) generated a permanent cell line (F cells) that produced a mutated (furin cleavage resistant) form of prM protein together with the E protein. Due to the mutation, these cells were unable to produce fusion competent M/E oligomers; however, they produced EPs containing the prM and the E proteins in large amounts. The F cell–produced EPs were recognized by a panel of monoclonal antibodies to JEV E protein, and they shared the biochemical property of empty viral particles produced by JEV-infected cells. Mice that received immunization with F cell–produced EPs emulsified with Freund's adjuvant failed to make JEV-neutralizing antibodies and these mice were only partially protected. The F cell–derived EPs, therefore, may not be suitable for vaccination, but these were shown to be useful as the antigen in JEV antibody detection ELISA.

Recombinant virus–based vaccines

Recombinant viruses represent a promising avenue for vaccine research both for improving existing vaccines and for developing new ones. Expression of antigens by virus vectors has proven to be a productive approach for studying *in vitro* and *in vivo* immune responses. A variety of viruses have been investigated as potential recombinant viruses. Although each viral vector has its own unique characteristics, an important feature of almost all recombinant viruses is the ability to induce not just humoral, but also cell-mediated immunity. The use of these recombinant viruses as vaccine candidates provides an advantage in that the foreign gene product is amplified during virus infection, thus increasing the antigen exposure. Foreign antigens thus expressed are processed and presented to the immune effector cells in a manner resembling natural infection. Pox and yellow fever viruses have been used for the development of a recombinant vaccine against JEV. The details of these systems are reviewed below.

Pox viruses expressing JEV proteins

The prototype of all recombinant viruses remains vaccinia virus. Yasuda *et al* (1990) and Konishi *et al* (1991) constructed vaccinia recombinants expressing JEV proteins. They found that recombinants that coexpressed the genes for the structural glycoproteins prM and E elicited high levels of neutralizing and hemagglutination-inhibiting antibodies in mice and protected the immunized animals from a lethal JEV challenge. However, vaccinia recombinant expressing the JEV nonstructural protein NS1, although induced anti-NS1 antibodies in immunized mice, provided only low level of protection against lethal JEV challenge (Konishi *et al*, 1991).

A recombinant vaccinia virus expressing the full-length E glycoprotein of JEV was compared for its antigenic structure and protective immunity in mice with a vaccinia virus expressing a strategically truncated E glycoprotein, containing approximately 80% of the N-terminal sequence (Jan *et al*, 1993). The truncation in the JEV E glycoprotein sequence corresponds to the position that has been shown to increase the immunogenicity of dengue type 4 or type 2 virus E glycoprotein (Men *et al*, 1991). Analysis of the JEV E glycoprotein in recombinant virus–infected cells showed that the C-terminally truncated protein retained an antigenic structure similar to the full-length E glycoprotein. The full-length E glycoprotein was detected predominantly intracellular, with a small fraction (<2%) present on the cell surface, whereas the truncated E exhibited an alteration in the intracellular transport pathway, resulting in increased accumulation (10% to 25%) on the cell surface and secretion (6% to 10%) into the medium. The C-terminally truncated E glycoprotein induced a greater antibody response and a higher level of protection in immunized mice than its full-length counterpart.

Due to the undesirable complications with recombinant vaccinia virus, highly attenuated recombinant vaccinia viruses have been favored as potential vaccines for human use. One such virus, NYVAC, was constructed by the deletion of 18 vaccinia virus open reading frames (Paoletti, 1996; Tartaglia *et al*, 1992). This virus is highly attenuated *in vivo*, but still induces an effective immune response comparable to that observed with the standard recombinant vaccinia virus (Tartaglia *et al*, 1992). NYVAC vectors encoding a wide range of pathogen-derived molecules have been constructed (Lanar *et al*, 1996; Tine *et al*, 1996). Keeping in view the safety concerns associated with the use of the vaccinia virus for human use, Konishi *et al* (1992) constructed recombinants of NYVAC expressing JEV proteins prM and E, with or without the NS1. Pigs immunized with these recombinant viruses synthesized JEV-neutralizing antibodies 7 days after the primary immunization and these antibodies increased after a booster dose. Significantly reduced viremia (serum JEV titers) was recorded in immunized pigs, compared to the

nonimmunized controls, when they were challenged with high dose of live JEV. These recombinants generated the cell-mediated immunity in mice in the form of the CTLs. The CTLs mainly recognized the E protein but did not recognize the NS1 protein, indicating that E protein's ability to generate the CTLs may be useful for the vaccine development.

Recombinant viruses have also been constructed from the modified vaccinia virus Ankara strain (MVA). This virus was generated by repeated passage in chick embryo fibroblasts, and it is replication deficient in mammalian cells (Moss, 1996). During the small pox eradication program, MVA was used extensively to vaccinate individuals at risk from the standard vaccinia vaccine, such as those with impaired immune systems. Nam *et al* (1999) constructed an MVA recombinant expressing the prM and the E proteins of JEV. Mice inoculated with the recombinant virus produced JEV-neutralizing antibodies and immunized mice were completely protected from a lethal JEV challenge.

Due to the safety concerns associated with replication-competent recombinant viruses, avipoxviruses have been examined as potential vaccines. These viruses abortively infect mammalian cells, while maintaining the capacity to present antigens to the immune system. Thus, these viruses provide a level of safety not obtainable with replication-competent viruses. Canarypoxvirus has received the most attention as a candidate recombinant vaccine, as it more efficiently induces immunity than other avipoxviruses (Taylor *et al*, 1991). ALVAC is a plaque-purified isolate of an attenuated vaccine strain of canarypoxvirus (Tartaglia *et al*, 1992). Mice immunized with an ALVAC recombinant expressing the prM and the E proteins of JEV produced virus-neutralizing antibodies and they were protected from a lethal challenge with JEV, demonstrating the potential of ALVAC as a vaccine vector (Konishi *et al*, 1994).

NYVAC-JEV and ALVAC-JEV, the two recombinant poxviruses expressing the prM and the E proteins of JEV, were evaluated in a controlled, randomized, double-blind clinical trial for safety and immunogenicity in human volunteers (Kanesa-Thasan *et al*, 2001). Groups of 10 volunteers who were vaccinia immune or vaccinia naïve were given two doses of each vaccine. The vaccines appeared to be equally safe and well tolerated, although the vaccinees had frequent occurrence of local warmth, erythema, tenderness, and/or arm pain after vaccination. There was no apparent effect of vaccinia immunity status on frequency or magnitude of local and systemic reactions. NYVAC-JEV elicited antibody responses to JEV antigens in vaccinia-naïve recipients only. ALVAC-JEV vaccine showed very poor immunogenicity. These data suggested that preexisting immunity to poxvirus vector suppressed antibody responses to recombinant gene products and hence alternate viral vectors for the antigen delivery need to be tested.

Yellow fever virus expressing JEV proteins

Yellow fever (YF) 17D is a live, attenuated vaccine that has been extensively used for human immunizations over the last 60 years, with superb record of safety and efficacy. Inoculation of a single dose of YF17D leads to generation of virus-neutralizing antibodies in nearly 100% of the vaccinees. The vaccine manufacturing procedure is well established and the vaccine is licensed for human use by the national control authorities worldwide. Thus YF17D would be an ideal vaccine vector for making recombinant vaccines.

Chambers *et al* (1999) constructed a chimeric virus ChimeriVax-JE by replacing the genes encoding two structural proteins (prM and E) of YF17D virus with the corresponding genes of an attenuated strain of JEV, SA14-14-2. Because ChimeriVax-JE structural proteins prM and E are of JEV origin, the immune response to vaccination is directed principally against JEV. Thus, C57Bl6 mice immunized with a single dose of increasing amounts of ChimeriVax-JE vaccine showed up to 100% protection when challenged with the lethal dose of JEV given intraperitoneally (Guirakhoo *et al*, 1999). Rhesus monkeys immunized with a single dose of the vaccine developed JEV-neutralizing antibodies. None of the immunized monkeys, when challenged with wild-type JEV, given intracerebrally, developed viremia or illness and had mild residual brain lesions, whereas sham-immunized control monkeys developed viremia, clinical encephalitis, and severe histopahological lesions in brain (Monath *et al*, 2000).

ChimeriVax-JE grew to high titers in diploid fetal rhesus lung (FRhL) cells, which are acceptable substrate for human vaccine production. Extensive safety studies on FRhL-grown ChimeriVax-JE have been carried out in mice and monkeys. None of the mice inoculated intracerebrally with graded doses of the vaccine showed symptoms of illness or died, whereas YF17D-inoculated mice died with lethal encephalitis (Monath *et al*, 2000). Similarly, none of the 10 rhesus monkeys inoculated intracerebrally with ChimeriVax-JE developed signs of clinical illness, whereas 4 out of 10 monkeys inoculated with YF17D developed signs of central nervous system dysfunction. Thus, both in mice and monkeys, ChimeriVax-JE was less neurovirulent than the YF17D vaccine. ChimeriVax-JE was restricted in its ability to infect and replicate in mosquitoes (Bhatt *et al*, 2000). The low viremia caused by ChimeriVax-JE in primates, and poor infectivity of mosquitoes, are safeguards against secondary spread of the vaccine virus.

Learning from the recombinant vaccinia immunizations in humans (as described above), a concern remained about the efficacy of ChimeriVax-JE in vaccinees already immunized with YF17D vaccine. A phase I/II clinical trial of ChimeriVax-JE in a limited number of individuals demonstrated that it induced JEV-neutralizing antibodies in 100% of naïve ($n = 6$) as well as YF17D-immune subjects ($n = 6$).

The vaccine was immunogenic and well tolerated in humans at doses of 10^4 and 10^5 plague-forming units (PFU) (Monath *et al*, 2002). These are encouraging observation that point to the potential of ChimeriVax-JE for human use as a vaccine against JEV. However, longevity of antibody response and generation of immunological memory against JEV are yet to be investigated as the human trials continue.

Plasmid DNA–based JEV vaccines

Nucleic acid immunization is the most recent approach to mobilizing the immune system against pathogenic invaders. The vaccine is usually a plasmid DNA capable of synthesizing a protective immunogen from a given pathogen. Direct injection of plasmid DNA containing open reading frames with appropriate eukaryotic transcription and translation control signals results in the synthesis of the immunogen in animals with conformation and post-translational modification patterns identical, in most cases, to those which occur during normal infection. Endogenous protein synthesis mimics viral infection in allowing presentation of foreign antigen by major histocompatibility complex class I, and uptake of soluble proteins by specialized antigen-presenting cells allows presentation by major histocompatibility complex class II.

In recent years, plasmid DNA vaccines have attracted a lot of attention for their ability to generate a broad range of immune responses, which include the induction of antibodies, generation of CD4+ helper T lymphocytes and CD8+ cytotoxic lymphocytes, and protection against a range of viral infections (Robinson and Torres, 1997). Besides, these vaccines are likely to be cheap, safer, and easy to produce. For JEV too, DNA vaccination has been attempted using plasmids capable of expressing the viral structural or nonstructural proteins. These attempts are reviewed below.

DNA vaccines expressing JEV structural proteins

The E protein appears to be the most suitable candidate antigen for DNA-based immunization against JEV. This follows from the fact that antibodies to the E protein neutralize JEV activity *in vitro* and the virus-neutralizing antibodies are protective against the disease. Besides, flavivirus E protein mediates membrane fusion and virus entry into the cell. Plasmids expressing E protein in the membrane-anchored, cytoplasmic, or secretary forms, with or without the prM protein, have been studied in mice for immunogenicity and protective efficacy. It was seen that intramuscular immunization of mice with plasmid DNA synthesizing the prM and the membrane-anchored E proteins of JEV elicited protective immunity in mice, 70% of the immunized mice survived after a lethal JEV challenge (Chang *et al*, 2000; Konishi *et al*, 1998). Mice immunized with the vector alone showed a low level (40%) of protection, suggesting a nonspecific adjuvant effect of the plasmid DNA. In another study using a plasmid encoding JEV prM and E protein, it was observed that two immunizations of mice by intramuscular or intradermal injections, at a dose of 10 or 100 μg per animal, elicited neutralizing antibodies at titers of 1:10 to 1:20 (90% plaque reduction), and all immunized mice survived a challenge with 10,000 LD_{50} of the P3 strain of JEV given intraperitoneally (Konishi *et al*, 1998). A single immunization with 100 μg of the plasmid DNA did not elicit detectable neutralizing antibodies but induced protective immunity. Spleen cells obtained from the immunized mice contained JEV-specific memory CTLs. These results indicated that the plasmid encoding prM and E proteins of JEV has the ability to induce a protective immune response against the virus, which includes JEV-specific antibodies and CTLs. Subsequently Chang *et al* (2000) showed that a single intramuscular injection of recombinant plasmid DNA encoding the JEV prM and E proteins was capable of eliciting JEV-specific antibodies, including neutralizing antibodies, which protected the immunized mice against lethal viral challenge.

Chen *et al* (1999) constructed a plasmid DNA encoding the full-length E protein, with 15 amino acids from the C-terminal end of the M protein serving as signal sequence. Immunization of mice with this plasmid generated high levels of protection against a lethal JEV challenge. The neutralizing antibody titers were, however, lower compared to the immunization with the live JEV. It has been shown that cosynthesis of prM is necessary for the proper folding, membrane association, and membrane assembly of the flavivirus E protein (Konishi and Mason, 1993; Lorenz *et al*, 2002). Because Chen *et al* (1999) did not have the prM protein in their construct, it is likely that the E protein produced by the plasmid was not processed and folded in the natural way. This may perhaps explain the low titers of the neutralizing antibodies induced by the vaccine. Striking differences were noted in the isotype profile and avidity of the specific antibodies induced by the intramuscular and gene gun immunizations using the DNA vaccine. Thus intramuscular immunization induced the Th-1 type of immune responses, whereas the gene gun immunizations induced the Th-2–type responses. Another interesting finding of this study was that the avidity of the anti-E antibody elicited by the intramuscular DNA immunization was significantly higher than that generated by the gene gun DNA immunization or the inactivated vaccine. It may be due to the adjuvant activity of the high dose of the DNA used in the intramuscular vaccination approach compared to the gene gun immunization.

Konishi *et al* (2000) carried out immunogenicity studies of JEV DNA vaccines in pigs, as this animal is an important amplifier of JEV in paradomestic environment. Pigs immunized with two doses of DNA vaccine (100 to 450 μg DNA), given 3 weeks

apart, generated neutralizing antibody titers of 1:40, which were higher than the 1:10 titer induced by the commercial formalin-inactivated JEV vaccine. Interestingly, serum antibody titers elicited by the DNA vaccine were higher in pigs than those observed in mice. In an effort to mimic natural infection, pigs immunized with DNA were then injected with the commercial JEV vaccine. This led to very high anamnestic response, indicating that DNA immunization was able to generate JEV-specific memory B cells.

Ashok and Rangarajan (1999) tested the immunogenicity of a plasmid expressing the intracellular form of the JEV E glycoprotein. The plasmid was able to raise a protective immune response in mice, although JEV-specific antibodies were not detectable. A significant postchallenge increase in T-cell proliferation was seen in the immunized mice, suggesting the ability of the vaccination-induced memory T cells alone to protect mice against the lethal challenge. An increase in the interferon (IFN)-γ expression and absence of interleukin (IL)-4 indicated the involvement of the Th-1 subset of the T-helper cells in conferring protection. The DNA construct used by Ashok and Rangarajan (1999) synthesized the E protein that lacked the signal sequence necessary for its proper transport and processing across the membrane. This may perhaps explain why no antibodies were detectable in mice immunized with the DNA construct. Based on the observations from all these studies, it is thus reasonable to speculate that plasmids expressing both prM and E proteins may serve as a better DNA vaccine candidate against JEV.

It can be seen from the above that the two forms of the E protein induced strikingly different immune responses: whereas the membrane-anchored E protein induced anti-E and anti-JEV neutralizing antibodies, the cytoplasmic form failed to induce detectable level of anti-JEV antibodies. It could be argued that the secreted form of the E protein may be a better immunogen, as it would be readily available for uptake by the antigen-presenting cells for its subsequent presentation on the class II major histocompatibility complex molecules. Indeed, vaccinia recombinant expressing the truncated form of the JEV E protein (consisting of about 80% of the N-terminal sequence) was found to be more immunogenic in mice (Jan *et al*, 1993).

We have studied the immunogenicity of the membrane-anchored and the secreted forms of the JEV E protein in mice (Kaur *et al*, 2002). DNA plasmids capable of synthesizing membrane-anchored or the secreted form of the JEV E protein together with the prM protein were delivered to mice by direct intramuscular injection or intradermally using a gene gun at a dose of 100 μg and 1 μg DNA per mouse, respectively. Immunogenicity and protective efficacy of the plasmid DNAs was compared with a formalin-inactivated, commercial JEV vaccine. Intramuscular plasmid immunization induced anti-E antibody titers similar to those induced by the formalin-inactivated vaccine. These titers were significantly higher than those obtained by the gene gun. The two forms of the E protein induced similar antibody titers by a given DNA delivery mode. Both plasmids delivered by intramuscular or the intradermal route generated high titers of JEV-neutralizing antibodies, although these titers were significantly lower than those induced by the vaccine. Spleen cells from mice immunized with plasmid DNA contained JEV-specific memory CTLs that were not observed in the vaccine immunized mice. Intramuscular DNA immunization resulted in a Th-1 type of immune response, whereas the gene gun–based delivery induced Th-2–type responses as indicated by the subclass of the anti-JEV immunoglobulin G (IgG) produced and the cytokines induced by the immunized mice spleen cells. Formalin-inactivated JEV vaccine induced a mixed Th-1, Th-2 response. Mice immunized with the plasmid DNAs showed about 60% protection in an intracerebral JEV challenge model. Form of the E protein or the route of DNA delivery did not affect the level of protection seen in immunized mice. Protection against JEV challenge in mice, as afforded by the plasmid immunization, was significantly lower than the over 90% protection afforded by the formalin-inactivated JEV vaccine.

Previously, Lin *et al* (1998) reported 70% protection against lethal JEV challenge of ICR mice immunized with plasmid DNA expressing the prM and the E proteins. Similarly, Konishi *et al* (1998) and Chang *et al* (2000) reported 100% protection of ICR mice immunized with plasmid expressing the prM and the E proteins. In all these cases, mice were challenged with a neuroinvasive strain of JEV administered intraperitoneally. In an intracerebral challenge model, about 38% to 51% protection was reported in mice immunized with plasmid DNA expressing the cytoplasmic form of the E protein (Ashok and Rangarajan, 1999). Interestingly, when mice immunized with plasmid used by Lin *et al* (1998) were challenged by intracerebral JEV inoculation, only 50% protection was observed (Ashok and Rangarajan, 2000). It would, therefore, appear that intracerebral challenge model is more demanding than the intraperitoneal challenge model. It should be noted that during the natural course of JEV transmission, the virus is injected directly into the blood stream of the host and so neither the intracerebral challenge nor the intraperitoneal challenge is a true replication of what may be happening in the nature. Because route of virus inoculation and its neuroinvasiveness will have major bearing on the course of immune response mounted by the host, the JEV vaccine efficacy, assessed in terms of mice protection, will vary according to the challenge system employed.

DNA vaccines expressing JEV nonstructural proteins
In addition to the structural proteins, the flavivirus NS1 is also expressed on the surface of the infected cells, besides being secreted in the extracellular environment (Rice, 1996). NS1 not only elicits

an immune response during the course of flavivirus infections but is also capable of conferring protection in experimental animal (Schlesinger *et al*, 1986). DNA immunization with the plasmid expressing JEV NS1 protein also elicited protective immunity in mice (Lin *et al*, 1998). Despite having no detectable neutralizing activity, the NS1 plasmid immunization elicited a strong antibody response exhibiting cytolytic activity against JEV-infected cells in a complement-dependent manner. 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 viral challenge. Biochemical analyses revealed that individually expressed NS1, but not NS1′, was readily secreted as a homodimer in large quantity and could also be efficiently expressed on the cell surface, as NS1′ lacked the membrane anchorage domains. This made NS1 significantly more immunogenic than NS1′ with respect to humoral responses and protective immunity induced in the immunized hosts, as NS1′ was likely to be retained in the endoplasmic reticulum rather than proceed along the secretary pathway (Chen *et al*, 1999; Lin *et al*, 1998). When NS1 and NS1′ coexisted in cells, the level of NS1 cell surface expression was much lower than in cells expressing NS1 alone. This implies that the presence of partial NS2A might have a negative influence on an NS1-based DNA vaccine. It was seen that immunization with DNA expressing NS1 alone was sufficient to protect mice against lethal JEV challenge (Lin *et al*, 1998). The other nonstructural proteins NS3 and NS5 were found to be ineffective in raising protective immunity (Chen *et al*, 1999). NS3 and NS5 proteins are thought to be involved in viral genome replication. It has been observed that the cell-mediated immunities, including both CD4+ and CD8+ T lymphocytes, are directed mainly against the conserved nonstructural viral proteins. NS3 and NS5 proteins are highly conserved amongst flaviviruses. Thus, T-cell responses to these two proteins should have played a significant role in protection but this was found not to be the case (Chen *et al*, 1999).

Conclusions

It can be seen that the prM, E, and NS1 proteins are the most effective candidates for inducing protective immunity against JEV. ChimeriVax-JE, the recombinant yellow fever virus expressing the prM and the E proteins of JEV has shown great potential as a JEV vaccine when tested in rhesus monkeys. Subsequent limited human trials have been encouraging because the vaccine induced JEV-neutralizing antibody response both in the naïve as well as YF17D-immunized individuals. In fact, JEV antibody levels were higher in YF17D-immunized animals than in the naïve subjects, dispelling concerns about antivector immunity. Although ChimeriVax-JE has proven to be safe in animals and also in limited human trials, its safety in large human population remains to be established and, whether ChimeriVax-JE would provide life-long immunity as in the case of YF17D, needs to be clarified. Nevertheless, the ChimeriVax technology holds great promise not only for the development of a recombinant vaccine against JEV but also against other flaviviruses such as Dengue and West Nile.

On the DNA vaccine front, different plasmids synthesizing various combinations of the prM, the E, and the NS1 proteins have shown their ability to induce protective immunity against lethal JEV infection in mice. In particular, intramuscular injection of plasmid DNA synthesizing the prM and the membrane-anchored E proteins has shown considerable potential as a possible JEV vaccine, as it generated strong Th-1 response coupled with the induction of IFN-γ synthesis in immunized animals. Although titers of the neutralizing antibodies induced by plasmid immunization were lower compared to the formalin-inactivated vaccine, these may perhaps be sufficient, as neutralization titers of 1:10 or more are commonly accepted as evidence of protection (WHO, 1998). Testing these constructs for induction of protective immunity against JEV in higher primates, such as rhesus monkeys, would be a logical step in the direction of developing a DNA-based recombinant vaccine against JEV. Although most DNA vaccines have generally been disappointing when tested in primates and humans, the JEV DNA vaccine has potential to succeed, especially because it has been found immunogenic in pigs. It may be of interest to mention that a DNA vaccine against West Nile virus, a flavivirus closely related to JEV, was recently found to be effective in horses where a single intramuscular injection of DNA encoding West Nile virus prM and E proteins induced protective immunity (Davis *et al*, 2001). Should JEV DNA vaccine be found effective in primates, it must be subjected to rigorous safety testing in preclinical trials, as concerns have been raised by regulatory agencies regarding their potential to integrate in the host genome, leading to a cancer-causing mutation and induction of autoimmunity.

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