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Review

The development of flavivirus vaccines

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Mosquito and tick-borne flaviviruses are the causative agents of some of the world's most important diseases, including dengue fever, yellow fever, Japanese encephalitis, tick-borne encephalitis and West Nile fever. Cumulatively, these viruses cause many millions of infections each year and impose a significant burden on public health resources, particularly in developing and newly developed countries. Vaccine development to eliminate flaviviral infections has been marked by uneven progress and a large number of setbacks. To date, no single approach has proved successful in leading to vaccine development against a wide range of flaviviruses, but the application of modern techniques to the problem is opening up new avenues of approach. This review summarizes some of the developments in vaccine research aimed at inducing protective immunity against flaviviral infections.

Key words: Attenuation, fever, encephalitis, flavivirus, hemorrhagic, vaccine.

INTRODUCTION

Flaviviruses, a group of positive single stranded RNA viruses are of significant concern as a major global health problem as they are the causative agent of a number of diseases affecting humans, and over 70 flaviviruses have been identified. Belonging to the family Flaviviridae, flaviviruses are predominantly transmitted to humans by the bite of infected mosquitoes or ticks. The clinical symptoms of flavivirus infections are generally classified into three major symptom groups; namely fever–rash, hemorrhagic fever with or without hepatitis and central nervous system diseases. West Nile virus (WNV), Japanese encephalitis virus (JEV), dengue virus (DENV), yellow fever virus (YFV), and tick borne encephalitis (TBE) virus are some of the significant members of this genus, all of which have an approximately 11kb RNA that encodes for three structural proteins and seven nonstructural proteins. Of the three structural proteins, the capsid (C) protein is involved in virion assembly, the precursor of membrane protein (prM) which is subsequently cleaved to membrane protein is involved in virion release and the envelope protein (E) is involved in virus entry and is the primary epitope generating neutralizing antibodies (Mukhopadhyay et al., 2006; Pugachev et al., 2005; Solomon and Mallewa, 2001).

Currently, despite significant research, there are neither broad spectrum anti-flaviviral drugs, nor drugs targeted to specific flaviviruses. Similarly, research directed against producing effective flavivirus vaccines has had only limited success, with the possible exception of YFV. The progress that has been made so far in the development of flavivirus vaccines, as well as the development of vaccines for YFV, JEV, DENV, WNV and TBEV will be briefly discussed.

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Abbreviations: WNV, West Nile virus; **JEV,** Japanese encephalitis virus; **DENY,** dengue virus; **YFV,** yellow fever virus; **TBE,** tick borne encephalitis; **C,** capsid; **prM,** precursor of membrane protein; **E,** envelope protein; **YE,** yellow fever; **LAV,** live attenuated virus; **FNV,** French viscerotropic vaccine; **Nab,** neutralizing antiboby; **YEC-AVD,** vaccine associated viscerotropic disease; **YEL-AND,** vaccine associated neurotropic disease; **JE,** Japanese encephalitis;**TrE,** truncated E; **ADE,** antibody dependant enhancement; **TBEV,** Tick-Borne Encephalitis Virus.

YELLOW FEVER VIRUS

YFV, the prototype flavivirus, is the causative agent of yellow fever (YF), which is a mosquito-borne hemorrhagic fever that can be found in the sub-Saharan Africa and tropical regions of Central and South America. It is estimated that approximately 200,000 cases occur

annually (of which 90% occur in Africa). Moreover, YF is also a threat to millions of people who travel to endemic areas (Monath, 2001; Robertson et al., 1996). The infection causes a broad spectrum disease including fever, hemorrhage, renal and myocardial injury and jaundice which leads to the term “yellow fever” disease. Although the early onset of the disease is treatable, there is no effective antiviral therapy once the infection is more advance (Roukens and Visser, 2008). In addition, the vector control strategies that used to work well in eliminating YF from many regions were either stopped or became ineffective, causing the reemergence of the disease. Therefore, vaccination appears to be the best method to control and prevent YF.

The development of YF vaccines began shortly after the isolation of YFV from the Ghanaian patient, Asibi, in 1927. The failure of inactivated vaccines earlier prompted researchers to focus on a live attenuated virus vaccine (LAV). The first effective YF vaccine, the French neurotropic vaccine (FNV), was developed by passaging the French viscerotropic virus, Dakar strain, through mouse brains (Sellards and Langret, 1932). However the FNV was discontinued in 1982 despite its high level of efficacy, due to the unacceptably high incidence of adverse effects in vaccines (Barnett, 2007). The second vaccine, YF-17D, was derived from the Asibi strain. The clinical isolated virus was repeatedly passaged on mouse and chicken embryonic tissues, which had nervous tissue striped off, resulting in the original YF-17D strain after 176 passages (Theiler and Smith, 2000). Initial vaccine production at different facilities was done without any standardization, leading to both over- and under-attenuation of the virus. To eliminate the inconsistency amongst vaccine lots, the World Health Organization (WHO) developed the seed lot system for the production of YF vaccine in 1945. The primary seed lot with a determined number of virus passages was established. All vaccine lots were prepared from a single passage from a secondary seed that had been characterized and substantially tested for safety (Monath, 2001). Currently, two sub-strains derived from YF-17D, 17DD and 17D-204, are used as YF vaccinees. YF-17DD, which is at passages 287 - 289, is used in Brazil, and YF-17D-204, which is at passages 235 - 240, is used in other countries where the vaccine is produced (Barrett and Teuwen, 2009). Both vaccine strains differ from the parental Asibi strain by 48 nucleotides, which results in 22 amino acid substitutions (dos Santos et al., 1995). The YF-17D vaccine is considered to be one of the most successful LAVs. A single dose of vaccine provides a protective level of neutralizing antibody (NAb) in 90% of the recipients within 10 days of immunization which increases up to 99% within 30 days (Monath, 2001). Although it has been shown that immunity can last longer than 30 years, the WHO requires a revaccination after 10 years (Barrett and Teuwen, 2009; Poland et al., 1981). After an estimated 200 million doses of YF-17D administered as of 2003, the vaccine is regarded as a highly safe vaccine (Marchevsky et al., 2003). Common adverse effects are generally mild and severe adverse effects are extremely rare. From 1945 to 2002, cases of YF vaccine associated neurotropic disease (YEL-AND) were reported in 23 patients worldwide with a preference of very young infants (Barnett, 2007). However, a more severe form of adverse event, vaccine associated viscerotropic disease (YEL-AVD), was first described in 2001.YEL-AVD is a pansystemic infection with a case fatality rate as high as 60%.Unlike YEL-AND, YEL-AVD is often found in the elderly (> 70 years), in which the risk increases up to 13.4 times when compared to young adult vaccinees (Roukens and Visser, 2008). Other vaccine adverse effects of concern are hypersensitivity reactions. Since all YF-17D vaccine produced from six manufacturers around the world is propagated in embryonated chicken eggs, individuals who are allergic to egg should not receive the vaccine (Barnett, 2007). Recently, the African green monkey kidney cell, Vero, has been proposed as an alternative for YF vaccine production (Souza et al., 2009).

JAPANESE ENCEPHALITIS VIRUS

One of the most important viral encephalitis causative agents in Asia is Japanese encephalitis virus (JEV) which is transmitted to human by *Culex* mosquitoes, principally *Culex tritaeniorhynchus* and *Culex quinquefasciatus*, which have fed on viremic animals, mostly domestic pigs. The geographic distribution of JEV is mainly in the Asian region including India, China, and all of South-East Asia, although outbreaks have also been reported in Pakistan and Australia. In endemic areas, at least 50,000 cases of Japanese encephalitis (JE) are reported annually, among which some 10,000 cases result in death. Because of the high mortality rate, the disease is considered as one of the most important health problems in the South-East Asia region. A typical illness starts with fever and other symptoms including vomiting and photophobia. Gradually the fever rises and encephalitis starts at which point the fatality rate is about 35%. Due to lack of effective medical treatment or a specific antiviral, attempts to develop a vaccine against the virus are considered a high priority (Barrett, 1997; Burchard et al., 2009; Kollaritsch et al., 2009).

Although vaccines against JEV have been available since the 1930’s, only one is currently in use internationally for vaccination of humans. This vaccine is a formalin inactivated whole virus prepared from mouse brains infected with the Nakayama strain of JEV. Internationally, this vaccine is licensed as JE-VAX. Despite widespread availability of this vaccine, it is comparatively expensive for large scale use in many Asian countries and there have been reports of severe allergic reactions to this vaccine (Plesner and Ronne, 1997). A second formalin inactivated whole virus vaccine from mouse

brain is produced by Biken for local use in Japan and is produced using the Beijing-1 strain, reflecting JEV strain diversity. Two further JEV vaccines, a cell culture derived inactivated virus vaccine using the P3 JEV strain and a live attenuated vaccine based on the neuroattenuated SA14-14-2 strain of JEV are prepared and used exclusively in China (Nalca et al., 2003). In addition, several plasmid based DNA vaccines against JEV have been reported. Amongst the JEV viral proteins, JEV E protein seems to be the most suitable one for plasmid DNA based vaccines, since antibodies against E protein are capable of neutralizing JEV activity. Plasmid constructs that contain JEV E protein together with the prM protein have been shown to provide a protective immune response to lethal JEV challenge in adult mice (Chang et al., 2000; Konishi et al., 1999). There are also DNA vaccines candidates utilizing non-structural proteins of JEV. Immunization of mice with plasmid DNA constructs containing JEV NS1 provided 90% protection against lethal challenge with JEV while plasmids bearing longer constructs failed to provide protection (Lin et al., 1998). However, vaccines utilizing JEV NS5 or NS3 failed to raise an effective immune response (Barrett, 1997; Kaur and Vrati, 2003; Liang et al., 2009; Mason et al., 2006; Nalca et al., 2003).

Recombinant virus based vaccines have also been reported in JEV vaccine development. JEV structural and non-structural proteins including prM, E, and NS 2B expressed in the vaccinia virus backbone can produce a protective immune response in mice (Konishi et al., 1991). Furthermore, a modified vaccinia virus, NYVAC, used to express the prM and E proteins of JEV with or without NS1 in pigs resulted in a significant loss of the viremic response in the animals (Konishi et al., 1992).In 1999, a chimeric vaccine between the YF-17D core and the prM and E proteins of the JEV attenuated vaccine strain SA14-12-2, was produced and was shown to be safe and effective and to produce a protective antibody response in mice and monkeys (Barrett, 1997; Kaur and Vrati, 2003; Liang et al., 2009; Mason et al., 2006; Nalca et al., 2003).

WEST NILE VIRUS

West Nile virus (WNV) is a mosquito-borne virus that causes a disease varying from asymptomatic to a febrile influenza-like illness and to lethal meningoencephalitis (Dauphin and Zientara, 2007). The name “West Nile”, as with many other viruses, derives from the location where it was first isolated, specifically the West Nile district in Uganda**.** The disease is endemic in Africa, Southern Europe, Russia, the Middle East, India and Australia and, from 1999, in North America. West Nile (WN) disease used to be considered a minor risk for human and horses. However during the past decade, many outbreaks have been reported in many countries. In the US alone, approximately 24,000 human cases (including almost 1,000 deaths) have been reported between 1999 and 2006 (Dauphin and Zientara, 2007). In addition, this newly reemerging disease appears to be more pathogenic with a higher incidence of severe neurological disease, especially in the elderly [CDC, 2003].

Currently, there is no licensed WNV vaccine for use in humans, although a DNA vaccine encoding the WNV, prM and E proteins, an inactivated cell culture derived whole virus and a canarypox-vector recombinant vaccine have all been approved for veterinary use in the US (Dauphin and Zientara, 2007; Pugachev et al., 2005). Several potential WNV vaccine candidates are under development for human use. A bacterial vector expressing domain III of WNV E protein has been proposed as a subunit vaccine candidate (Chu et al., 2007; Martina et al., 2008). Despite the high titer of NAb and protection in a murine model, the unlicensed adjuvant and lengthy vaccination schedule appear to be significant obstacles for the bacterial expression system (Widman et al., 2008). A more promising subunit vaccine is the recombinant truncated E (trE) and NS1 proteins expressed in *Drosophila* S2 cells. The high yield of the system and the excellent efficacy profile of the vaccine make it one of the best flavivirus subunit vaccines (Lieberman et al., 2007; Watts et al., 2007). Following the development of the licensed equine DNA vaccine, the human version of a DNA vaccine encoding WNV, prM and E showed the induction of NAb in a phase I clinical trial (Martin et al., 2007). A number of LAVs for WNV have exhibited outstanding results in animal models and clinical trials. The live attenuated Schwarz strain of measles virus expressing the secreted form of E protein from a virulent strain of WNV induced high level of NAb and showed protection against a lethal challenge of WNV in mice (Despres et al., 2005). Preclinical trials of a live attenuated WNV/ Dengue4 chimeric virus showed moderate to high titers of NAb in a non-human primate model (Pletnev et al., 2003). Nonetheless, the most advanced LAV for WNV is ChimeriVax-WN02, a live attenuated recombinant chimeric YF-17D backbone with WNV prM and a mutated E. This vaccine demonstrated an exceptional efficacy and safety in both preclinical and phase I clinical trials, with the phase II trial scheduled to be completed this year (Arroyo et al., 2004; Hall and Khromykh, 2007). Other vaccine approaches that have shown promise in mouse models include a live attenuated WNV isolate and RepliVax WN, a defective pseudoinfectious WNV lacking a functional C gene (Mason et al., 2006; Widman et al., 2008; Yamshchikov et al., 2004).

DENGUE VIRUS

Dengue virus (DENV) is the causative agent for a febrile mild disease called dengue fever to the more severe, and in some case lethal forms, dengue hemorrhagic fever and

dengue shock syndrome. The virus includes four closely antigenically related serotypes termed DEN-1 to DEN-4 that are transmitted to humans by the bite of infected female mosquitoes belonging to *Aedes* (predominantly *Aedes aegepti* and *Aedes albopictus*) family. Dengue is distributed in tropical and sub-tropical areas including South-East Asia. Annually, more than 100 million people are reported to become infected with the dengue virus and approximately 24,000 fatal cases are reported (Malavige et al., 2004; Solomon and Mallewa, 2001).

Infection with one dengue serotype induces lifelong immune protection against reinfection with the homotypic serotype, but not to the other serotypes. Subsequently second, third and even fourth infections with heterotypic serotypes can occur, and these are frequently associated with the more severe forms of the disease. The increased severity of subsequent dengue infections is believed to occur as a result of the process termed antibody-dependant enhancement or ADE in which existing antibodies against one serotype act as non-neutralizing anti-DENV antibodies facilitating entry of the virus to FC receptor carrying cells such as monocytes. Large amounts of research have been undertaken to develop a successful vaccine against DENV which are still ongoing. Because the protective immune response must be raised simultaneously against all four dengue serotypes to avoid complications as a result of ADE, vaccine development for DENV has encountered significant problems (Rabablert and Yoksan, 2009; Williams et al., 2009).

Two live attenuated DENV vaccines have been developed so far. The first one, developed by the Walter Reed Army Institute of Research (WRAIR), is a tetravalent LAV produced by serial passage in primary dog kidney cells with a final passage in fetal rhesus monkey lung cells. While monovalent formulations generally showed good seroconversion in volunteers, difficulties were encountered in balancing the attenuation of the four component candidates (Rabablert and Yoksan, 2009; Wilder-Smith and Deen, 2008).The other live attenuated DENV vaccine was produced in Mahidol University, Thailand, and was licensed by Aventis Pasteur (now, Sanofi Pasteur). For DEN-1, DEN-2 and DEN4, the vaccine was produced by passage in primary dog kidney cells (PDK) while DEN-3 was derived by serial passage in primary African green monkey kidney cells. In a similar situation to the WRAIR LAV candidate vaccine, monovalent vaccines showed good seroconversion but tetravalent formulations encountered difficulties with regards to the lack of a balanced immune response to each of the four components. In addition there was evidence of systemic symptoms in recipients of the tetravalent formulations. Currently, development of these two LAV dengue vaccines is suspended (Rabablert and Yoksan, 2009; Wilder-Smith and Deen, 2008). Both of the live attenuated candidates were developed with little or no knowledge of the attenuating mutations, due in part to the development of the vaccines as strains rather than as clones. The National Institute of Allergy and Infectious Diseases (NIAID) and the National Institute of Health (NIH) in the US have employed a strategy of introducing defined attenuating deletions in the 3'-untranslated regions of full length dengue clones. Tetravalent formulations have been shown to be immunogenic and protective in monkeys, and further trials are ongoing.

Current chimeric vaccine candidates include YFV/dengue and dengue/dengue chimeras. The ChimeriVax candidate vaccine uses the YFV vaccine (YF-17D) as a backbone and replaces the prM and E genes with the respective genes from all four dengue serotypes, as is also being undertaken with WNV and JEV vaccine development. Tetravalent formulations have been shown to provide protection against virulent virus challenge in monkeys, and the monovalent DEN-2 ChimeriVax has been shown to be safe and immunogenic in humans. Clinical trials of this vaccine candidate are ongoing (Webster et al., 2009; Wilder-Smith and Deen, 2008). The US CDC has developed a set of chimeric dengue/dengue vaccine candidates based upon the Mahidol University cloned DEN-2 PDK-53 vaccine candidate. The candidate chimeric vaccines contain the structural proteins of each of the serotypes (C, prM, and E) together with the DEN-2 PDK-53 nonstructural proteins and ancillary sequences. The candidates have been shown to be immunogenic in mice and protective against virulent virus challenge (Wilder-Smith and Deen, 2008).

Both LAV candidates and chimeric vaccine candidates use replication competent viruses which carry a small, but not zero, risk of reverting to a more pathogenic phenotype. In addition, balancing the immune response to each component of the vaccine has proven difficult with live attenuated and chimeric vaccine candidates. The use of whole inactivated virus vaccines could overcome these two disadvantages. However, studies with inactivated viruses suggest that the immune response is less robust and that multiple booster doses as well as the use of adjuvants are required to generate long term immunity. The use of adjuvants, coupled with the low titers of dengue grown in cell culture suggests that inactivated whole virus vaccines could be significantly more expensive to produce than vaccines by alternate technologies (Webster et al., 2009). However, the uniformity of the immune response to the different serotypes suggests that this vaccine might have a role for specific situations where other dengue vaccines are deemed inappropriate.

A possible compromise between whole inactivated virus vaccines and LAV candidates may lie in the use of replication incompetent vaccines. Based on the RepliVax platform, Suzuki and colleagues have (Suzuki et al., 2009) substituted the prM and E proteins in this repli-cation incompetent WNV vaccine candidate with the corresponding genes from DEN-2 and this construct is currently undergoing evaluation. Further experimental approaches currently undergoing evaluation include

recombinant subunit vaccines, nucleic acid vaccines and live vectored vaccines. Recombinant subunit vaccines currently undergoing investigation include purified whole or truncated dengue E protein (Guzman et al., 2003; Robert Putnak et al., 2005), a consensus domain III of the dengue envelope protein (Leng et al., 2009) and a chimeric tetravalent vaccine consisting of all four serotype specific E protein domains III in tandem (Etemad et al., 2008). As with the whole inactivated virus vaccine candidates, subunit vaccines require the use of adjuvants and the combination of subunit purification and the adjuvant may make these vaccines comparatively expensive. Live vectored vaccine candidates have primarily used vaccinia virus, pox virus, adenovirus or more recently the measles vaccine virus as a vehicle to deliver the antigen, but as yet there is little data on the suitability of these as potential commercial vaccines. A few potential DNA vaccines are in development and while these elicit suitable antibody responses in animal studies, these vaccines are likely to need sophisticated and expensive immunization regimes, which would tend to minimize their suitability for large scale use in developing countries (Webster et al., 2009).

TICK-BORNE ENCEPHALITIS VIRUS

TBEV is the etiological agent of tick-borne encephalitis disease (TBE), whose presentation ranges from a febrile illness to a potentially fatal CNS infection that leads to long-term neurological sequelae in up to 50% of the surviving patients (Widman et al., 2008). TBEV is transmitted primarily by Ixodid ticks and is endemic in almost all the European countries and the Far East. There are three subtypes of TBEV, the European, Siberian and Far-eastern, in which the degrees of virulence are different (Mansfield et al., 2009).

The first licensed TBE vaccine is a whole inactivated virus vaccine and was first created in Austria in 1971. The vaccine, FSME-IMMUN®, was prepared from a TBEV isolate (Neudorfl strain) which was grown in primary chicken embryo cells followed by formaldehyde inactivation and purification by gradient ultracentrifugation. Subsequently, a number of other European countries adapted the same concept to produce variations of the TBE vaccines using different virus isolate strains (Barrett et al., 2003). Vaccination against European subtypes appears to be protective against Far-eastern subtypes as well since cross reactive antibodies can be detected (Leonova et al., 2007). To improve the safety profile of these vaccines, vaccine production was continuously modified by removal of additives and biological remnants from virus propagation (Barrett et al., 2003). Although these vaccines show an excellent safety and efficacy profile, 10,000 hospitalized cases are reported annually due to low vaccination rates in high-risk areas, primarily because of the requirement for multiple boosters and the high cost of the vaccine (Widman et al., 2008). Other types of TBE vaccine under development include truncated E protein or NS1 subunit vaccines, LAVs such as recombinant vaccinia virus expressing TBE-NS1, chimeric flavivirus vector/TBEV, C protein deleted TBEV and nucleic acid based vaccines (Aleshin et al., 2005; Pugachev et al., 2005; Widman et al., 2008).

CONCLUSION

Many human pathogenic flavivirus are seeing a signifycant increase in occurrence as a result of either emergence or re-emergence of the virus, particularly in developing and newly developed nations of the world. As such, the need for cheap, safe and effective vaccines to combat these diseases is increasing. Despite the early and significant success with Yellow fever vaccines, this has failed to translate into a broad array of effective vaccines directed to other flaviviruses. The classic methodology of generating live attenuated viruses through serial passages has encountered significant problems, most notably with dengue where the phenomenon of ADE serves to dramatically complicate the generation of an effective vaccine, given that simultaneous and equal protection against all four serotypes is a significant requirement. Novel techniques including subunit vaccines, chimeric vaccines and DNA vaccines offer some hope, but overall progress has been slow. It is hoped that newer technologies, when completely validated and bought to the market will be able to be adapted quickly, cheaply and effectively to other members of the flavivirus genus.

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