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Yellow fever vector live-virus vaccines: West Nile virus vaccine development

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By combining molecular-biological techniques with our increased understanding of the effect of gene sequence modification on viral function, yellow fever 17D, a positive-strand RNA virus vaccine, has been manipulated to induce a protective immune response against viruses of the same family (e.g. Japanese encephalitis and dengue viruses). Triggered by the emergence of West Nile virus infections in the New World afflicting humans, horses and birds, the success of this recombinant technology has prompted the rapid development of a live-virus attenuated candidate vaccine against West Nile virus.

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Yellow fever (YF) virus is a positive-strand RNA virus widely used as an attenuated live-virus vaccine. Other RNA live-virus vaccines include measles, mumps, rubella and poliovirus, all of which have been in use for many years with remarkable safety and efficacy profiles[1,2], although this has been challenged by recent reports linking measles virus or measles vaccines with the occurrence of juvenile CROHN'S DISEASE (see Glossary) and autism[3–5]. Recently, some of these RNA viruses have been explored as vectors to deliver foreign genes. A promising example is influenza virus, a negative-strand RNA virus commercialized as a nonliving-virus vaccine, first used in 1989 as a vaccine vector. Influenza vector technology, based on delivering defined EPITOPES by substitution of surface residues of the influenza spike proteins, exploits influenza TROPISM to target generation of MUCOSAL IMMUNITY in the upper and lower respiratory tracts[6], as well as systemic responses, which result in immunity in genital and intestinal tracts[7]. Poliovirus, a positive-strand RNA virus like YF, has been taken a step forward in preclinical studies and tested as a vaccine vector genetically modified to induce immunogenicity against tetanus toxin and simian immunodeficiency virus[8,9]. Most advances regarding the use of RNA viruses as delivery systems rely on the significant progress of RT-PCR, REVERSE GENETICS, plasmid vectors and *in vitro* transcription systems. The first infectious animal RNA virus clone to be recovered from a full-length cDNA molecule was the 7.5-kb poliovirus in 1981[10]; only last year, a 27-kb porcine coronavirus, the longest viral RNA genome known, was successfully cloned using a bacterial artificial chromosome (BAC), a low-copy-number plasmid[11]. The instability of bacterial vectors carrying viral cDNA was a gigantic hurdle for this technology, as experienced during the YF virus cloning. Rice *et al.*[12] were the first to generate YF virus RNA from a pair of cDNA clones ligated *in vitro* before RNA transcription. The same technology was

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later used by Thomas J. Chambers to create a chimeric virus where the sequence of YF envelope genes was substituted with the sequence from Japanese encephalitis (JE), a member of the same group of viruses known as flaviviruses[13]. Chambers' chimeric concept originated from work in 1992 where another pair of flaviviruses (i.e. tick-borne encephalitis and dengue) was used to create a chimeric live virus with vaccine potential[14]. Pletnev's dengue chimera encodes the structural genes of a virulent tick-borne encephalitis strain, while retaining NEUROVIRULENCE in a mouse model. Attenuation was achieved owing to loss of peripheral invasiveness, thus creating a conceptual method for attenuating flaviviruses. Following these principles, the chimeric YF vaccine technology has evolved to create a platform, now delivering structural genes encoding sufficient protective antigens to produce live-attenuated candidate vaccines against JE, dengue[15] and more recently, West Nile (WN).

Yellow fever live-attenuated vaccine

YF 17D vaccine strain was developed 65 years ago by empirical methods, which included a substantial passage history[16]. The vaccine is used for wide-scale immunization of children in tropical areas, travelers and military personnel. This long experience in >350 million people has provided assurances of safety and efficacy, making yellow fever an ideal vector for foreign genes. However, there are disadvantages to this practice. As with any other positive-strand virus, the high rate of genetic variation caused by viral replication by polymerases without proofreading enzymes and mutations involved in adaptation to different host cells could lead to unexpected surprises. Replacing the structural genes of YF 17D with those of other flaviviruses might alter tropism, and the potential of replication in unanticipated tissues will have to be examined individually. On the other hand, a significant advantage of a live vaccine includes the development of rapid and durable HUMORAL and cell-mediated immune responses that closely mimic those directed against the wild-type virus.

Chimeric vaccine construct technology

YF and WN are members of the genus *Flavivirus* – enveloped, positive-strand RNA viruses of approximately 11 kb, which are transmitted by arthropods, like ticks and mosquitoes[17]. WN virus was first isolated from a febrile adult woman in the West Nile district of Uganda in 1937[18,19]. The virus was not recognized as a cause of severe meningoencephalitis until an outbreak in Israel in 1957[20]. After appearing over subsequent years in Europe, Asia and Australia, where it afflicted humans and horses, WN made its appearance in North America in 1999[18]. Since the first recorded outbreak in the western hemisphere, WN has become part of the prevention and control mandate of several government agencies. The availability of a technology (ChimeriVax) based on the use of YF 17D as a vector to deliver effective immunity against JE virus, a close relative of WN virus, prompted the development of a WN vaccine. A formalin-inactivated JE vaccine (JE-VAX®, Aventis Pasteur) is available in the USA and marketed to travelers. Although immunity to JE might provide cross protection against WN in macaques [21], there are insufficient data to recommend the use of the JE-VAX® to protect humans and horses.

The development of the WN vaccine followed the path drawn by the development of a live-attenuated YF–JE chimeric vaccine (ChimeriVax-JE)[13,22]. The same two-plasmid system used to develop ChimeriVax-JE was utilized by replacing the sequences for the JE *prM* and *E* genes with those of the WN NY-99 strain virus (Fig. 1). The resulting VIRION has the envelope of WN, containing structures involved in virus–cell attachment and virus internalization, all antigenic determinants for neutralization, and epitopes for T-cell mediated immunity. The capsid protein, nonstructural proteins and non-translated termini (UTR) responsible for virus replication remain those of YF 17D vaccine. Like ChimeriVax-JE, the chimeric YF/WN virus replicates in tissue culture to titers in excess of 7 $\log_{10}$ plaque-forming units (PFU)ml^{-1}. The chimeric virus is expected to replicate efficiently in the host as well as provide protective immunity against WN virus.

Glossary

Crohn's disease: A chronic inflammatory disease, primarily involving the small and large intestine, but which can affect other parts of the digestive system as well.
Cytotoxic T cell: T cell that can kill other cells. Most cytotoxic T cells are MHC class I-restricted $CD8^+$ T cells, but $CD4^+$ T cells can also kill in some cases. Cytotoxic T cells are important in host defense against cytosolic pathogens.
Epitopes: Refers to a site on an antigen recognized by an antibody; epitopes are also called antigenic determinants. A T-cell epitope is a short peptide derived from a protein antigen. It binds to an MHC molecule and is recognized by a particular T cell.
Humoral immunity: Specific immunity mediated by antibodies made in a humoral immune response.
Mucosal immunity: Resistance to infection across the mucous membranes. Mucosal immunity depends on immune cells and antibodies present in the linings of reproductive tract, gastrointestinal tract and other moist surfaces of the body exposed to the outside world.
Neuroinvasive: A virus capable of invading the central nervous system upon peripheral inoculation.
Neurovirulence: The ability of a virus to replicate in brain tissue and cause encephalitis.
Reverse genetics: A strategy for studying gene structure and function by site-directed mutagenesis. The modified nucleic acid can then be introduced into an organism to study the effect of the mutation.
RT-PCR (reverse transcriptase polymerase chain reaction): PCR in which the starting template is RNA, implying the need for an initial reverse transcriptase step to make a DNA template, followed by separate conventional PCR.
Tropism: Tissues or host cells in which a virus can replicate.
Virion: A virus particle existing freely outside a host cell.

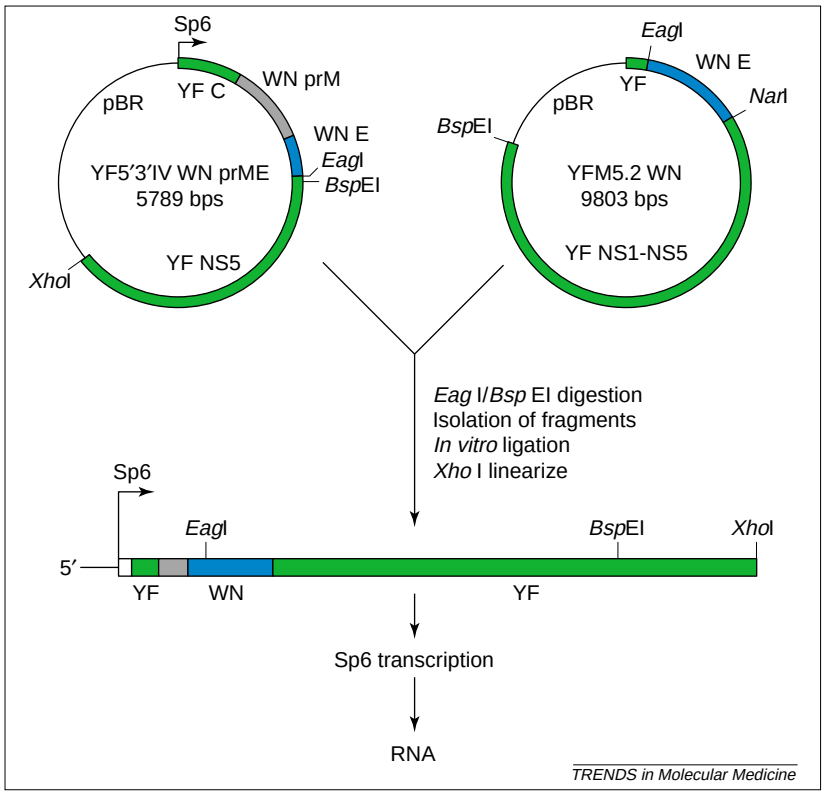

Fig. 1. Two-plasmid system encoding the YF–WN chimeric vaccine. Plasmid YF5′3′IV WN preMembrane and envelope protein genes (*prME*) encodes the 5′ UTR, yellow fever capsid (YFC), West Nile virus prM (gray) and 5′ end of E (blue), and the 3′ end of yellow fever NS5 and UTR. Plasmid YFM5.2 WN encodes the second half of E (blue) and the non-structural genes of yellow fever NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5. West Nile *prME* gene fragments were amplified by RT-PCR and subcloned into the two-plasmid system by overlap-extension PCR. Silent *Eag* I and *Bsp* EI sites were introduced for *in vitro* ligation steps necessary to create a full-length cDNA before *in vitro* transcription. Naked RNA initiates productive infections after transfection of a Vero cell line.

ChimeriVax-JE vaccine: immunological basis for protection, efficacy and safety

Neutralizing antibodies are the first line of defense against flaviviruses. In a live vaccine, virus replication induces a fast and durable response. CYTOTOXIC T CELLS eliminate viruses that were able to establish intracellular infections. This defense mechanism recognizes the E protein as well as nonstructural proteins, making infected cells a target for cell killing[23]. Exposure to the virus by natural infection post-vaccination would lead to a rapid secondary response, stronger than the primary response, and increased cross-reactivity with other members of the *Flavivirus* genus encoding similar epitopes. Studies in mice[24] and monkeys[25,26] were conducted to determine the ability of ChimeriVax-JE vaccine to protect animals against challenge. Challenge of both immunized and control monkeys with JE virus was performed by an intracerebral (IC) inoculation of 5 $\log_{10}$ PFU. Natural JE infection occurs by peripheral inoculation of small doses of virus in mosquito saliva; it was estimated by *in vitro* methods that a female mosquito releases about 2 $\log_{10}$ PFU while blood feeding[27]. Typically, rhesus monkeys do not develop brain infection and encephalitis after peripheral JE virus inoculation. The IC inoculation disease model is thus used in vaccine efficacy tests. Therefore, relative to peripheral natural infection, IC inoculation with 5 $\log_{10}$PFU is a severe test of protection. All ChimeraVax-JE immunized rhesus developed high titers of neutralizing antibodies after subcutaneous vaccination. Following IC challenge with a wild-type JE virus, vaccinated monkeys were 100% protected against viremia and clinical encephalitis providing clear evidence of vaccine efficacy[26].

Because the neurotropic nature of these viruses might lead to encephalitis in humans, the safety of live JE and WN vaccines has to be carefully addressed. *Flavivirus* genome replication lacks proofreading activity, resulting in a significant rate of mutations. For example, one single codon alteration from GAA (glutamic acid) to AAA (lysine) in the envelope protein gene markedly reduced the virulence of a JE virus isolate[28]. To study the genetic stability of a ChimeriVax vaccine construct, the virus was passed six times in brain tissue of mice and up to 18 times in cell culture. For ChimeriVax-JE, the vaccine genome and attenuated phenotype were shown to be stable on passage[24]. The molecular basis of attenuation of ChimeriVax-JE vaccine was studied by systematic mutagenesis[22]. Neurovirulent JE strains and attenuated JE strains were compared by sequence analysis, and amino acid residues in the *prME* sequence of the vaccine that were implicated in virulence were reverted. The elucidation of multiple attenuation determinants in the *E* gene provided a rationale for the development of a chimeric WN vaccine.

Molecular basis of ChimeriVax-JE vaccine attenuation and the rationale for WN vaccine development

In principle, the attenuated phenotype and safety profile of the ChimeriVax-JE virus are based on the derivation of all its genome from proven vaccine strains (i.e. YF 17D and JE SA14-14-2). The live-attenuated JE SA14-14-2 vaccine is used only in China and possesses an excellent safety record[29]. The basis of attenuation lies within the genome sequence of these RNA viruses. Sequence changes affect the phenotype by altering the function of the gene products, the secondary structure integrity of the RNA molecules, or both. The effect of the sequence of the *prME* genes of JE on its virulence phenotype was revealed following mouse studies that compared ChimeriVax-JE with a corresponding neurovirulent YF/JE Nakayama construct[13]. The study showed lack of neurovirulence for ChimeriVax-JE relative to the Nakayama construct or to a YF 17D vaccine control. The E proteins of wild-type and SA14-14-2 (attenuated) JE strains differ in ten amino acid residues (Table 1). A rational sequence reversion study to understand the basis of ChimeriVax-JE attenuation provided additional insight. Envelope amino acid residue E138 had a dominant effect, being required for reconstitution of neurovirulence in a mouse model,

Table 1. Mutagenesis target residues for West Nile vaccine attenuation

Virus	Amino acid									
	107	138	176	177	227	244	264	279	315	439
West Nile$_{NY99}$	L	E	Y	T	S	E	Q	K	A	K
JE wild type	L	E	I	T	P[a]	E	Q	K	A	K
JE$_{SA14-14-2}$ strain	F	K	V	A	S	G	H	M	V	R

[a]E227 was found to be a proline (P) in Japanese encephalitis (JE) Nakayama (wild-type) strain.

whereas E107, 176, 279, 264 and 227 residues, in that order, only contributed to measurable increases in neurovirulence[22].

WN virus is a member of the JE antigenic complex, a subgroup of very closely related flaviviruses defined by cross-neutralization[30]. The sequence similarity of the E protein of WN and JE is shown in Fig. 2. Because of the high degree of similarity, it is hypothesized that the introduction of mutations linked to the attenuation of JE SA14-14-2 will also attenuate ChimeriVax-WN. For ChimeriVax-WN, the approach will be to systematically mutate the sequence of the wild-type WN NY99 strain E protein to assess the effect of single mutations at the equivalent of residues E138, 107, 176 and 279. Additional mutagenesis targets could be considered from the outcome of other *Flavivirus* studies. Mutations in the vicinity of E315 are associated with altered virus tropism and changes in virulence[31–33]. Position E244 might not play a significant role as it is either a glycine (G) or glutamic acid (E) in several virulent JE strains analyzed[34,36]. Position E439

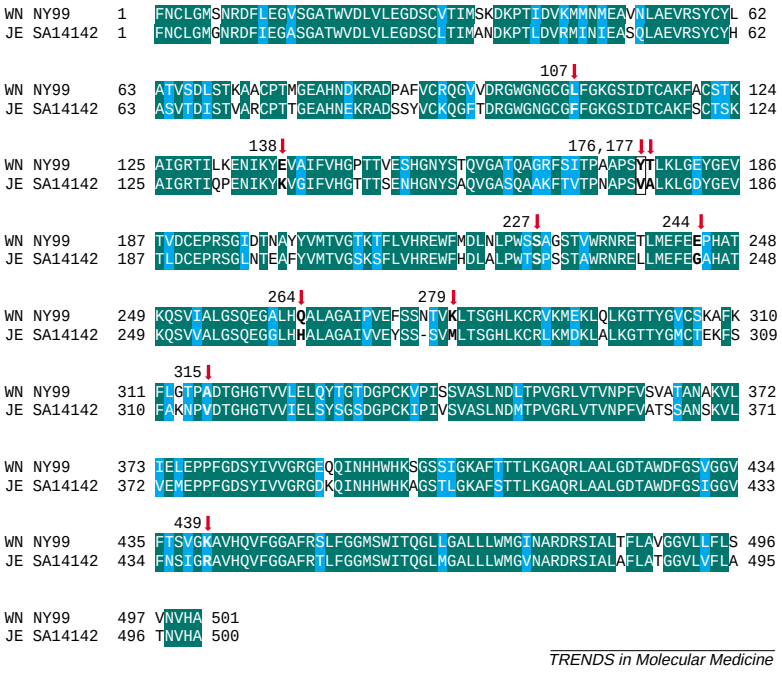

Fig. 2. Sequence alignment of the E protein of WN NY-99 strain and the JE SA14-14-2 vaccine strain. Identical and conserved residues are shown in blue and green, respectively. A 77% identity was predicted by CLUSTAL W alignment. Red arrows map the location of ten amino acid residues that distinguish virulent JE Nakayama strain E protein (see Table 1).

represents a conserved K→R substitution in the transmembrane region of the E protein with very little chance of any major effect. A tyrosine (Y) at position E176 (Table 1) might contribute to the attenuation of ChimeriVax-WN in its present configuration. The hypothesis will be tested by targeted mutation of E176 to isoleucine and testing for an increase in neurovirulence. Eventually, combinatorial mutation studies will define an attenuated vaccine.

Surprisingly, the chimeric ChimeriVax-WN construct containing wild-type WN *prME* was found to be significantly less neurovirulent than YF 17D vaccine. Young adult mice (five per group) inoculated IC with graded doses between 2 and 6 $\log_{10}$ PFU resulted in scattered deaths (ranging from 20–60% mortality) without a clear dose effect, perhaps owing to the susceptibility of the model used. By contrast, YF 17D was 100% lethal at doses >1 $\log_{10}$ PFU in the same mouse model. In addition, ChimeriVax-WN has lost the NEUROINVASIVE property typical of wild-type WN. Virus replication in brain tissue or viremia levels in moribund animals was not measured. However, the average survival times (nine days) were similar for both viruses. The data suggest that the sequence-unmodified chimera might be a vaccine candidate. Ultimately, the safety of a live attenuated ChimeriVax-WN will rely on sequence stability of the genome particularly at the E protein amino acid positions identified to play a role in attenuation.

Additional WN vaccine approaches

The biotech company Baxter/Immuno (Austria) has initiated efforts to develop a formalin-inactivated human vaccine. Fort Dodge Animal Health (USA) has initiated development of both a formalin-inactivated and a DNA plasmid vaccine for horses. The DNA technology was developed at the Centers for Disease Control and Prevention, Ft. Collins (CO, USA) and a study in horses at Colorado State University demonstrated protection against virus challenge[35]. Lustig *et al.* produced a live attenuated WN virus isolate derived from empirical passage of a wild-type strain in *Aedes aegypti* mosquito cells. One dose of the attenuated virus showed 100% protection in both mice and geese IC inoculation disease models challenged with a homologous wild-type WN (Ref. 37). A formalin-inactivated WN vaccine should work as effectively as JE-VAX® against JE. However, requiring multiple doses for efficacious protection, this vaccine would not be beneficial in an immediate threat of epidemic disease. A DNA vaccine for expression of WN *prME* genes only might be protective for horses but is likely to require multiple doses. Considering the possibility of a spontaneous virulent reversion owing to the relatively high mutation rate of RNA viruses, a live attenuated full-length WN vaccine is not as safe as the WN *prME* DNA vaccine. Furthermore, the WN *prME* DNA vaccine will produce antibodies only against the WN

membrane proteins encoded in the plasmid thus the response to nonstructural viral genes, typical of natural WN infections, will be absent. This will facilitate screening of traded horses by the existing antibody detection methods. A chimeric live vaccine (ChimeriVax-WN) encoding the nonstructural genes of YF 17D might be as effective in horses as the DNA vaccine. However, it has to be determined whether the vaccine will replicate and prove effective.

Conclusions

The need for a WN virus vaccine will be defined by the progression of WN outbreaks. The most promising candidates for a human vaccine include an 'old technology' formalin-inactivated virus vaccine, and a live chimeric vaccine where a rational approach is used to create a safe recombinant vaccine. A live vaccine that rapidly elicits immunity after a single dose would be much preferred over a multi-dose product for use in an impending epidemic, as surveillance of birds and mosquitoes provides only a brief warning of virus activity before the risk of human disease. The ecology of WN virus in the western hemisphere is still evolving. At least five species of mosquitoes were found to be competent vectors in experimental transmission studies with the WN NY99 strain[18]. Further understanding of the basic transmission cycle of WN in birds and mosquitoes is needed to implement proper control mechanisms. Ultimately, clear definition of the risks associated with WN virus epidemics might help create sufficient preventive measures to avoid the use of a vaccine. In the meantime, for as long as outbreaks continue to occur predominantly in urban areas where media attention is so ubiquitous, and with a system that relies on sightings of dead crows to forecast a potential WN epidemic, the need to develop a vaccine will continue.

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