rearrangement during passage of new particles towards the cell surface through acidic secretory compartments (Guirakhoo et al., J. Gen. Virol. 72:1323-1329, 1991; Guirakhoo et al., Virology 191:921-931, 1992).

ChimeriVax™ technology has been used to create live, attenuated vaccine candidates against medically important Flaviviruses. It employs the YF 17D vaccine virus as a vector in which the prM-E genes are replaced with the prM-E genes from a heterologous Flavivirus, such as JE, dengue, West Nile, or St. Louis encephalitis viruses (Monath et al., Vaccine 17:1869-1882, 1999; Monath et al., Curr. Drug Targets - Inf. Disorders 1:37-50, 2001; Monath et al., Vaccine 20:1004-1018, 2002; Guirakhoo et al., Virology 257:363-372, 1999; Guirakhoo et al., J. Virol. 75:7290-7304, 2001; Guirakhoo et al., Virology 298:146-159, 2002; Pugachev et al., Int. J. Parasitol. 33:567-582, 2003; Guirakhoo et al., J. Virol. 78:4761-4775, 2004). Previously, the ChimeriVax™-JE vaccine virus, containing the prM-E genes from the SA14-14-2 virus (live attenuated JE vaccine used in China), was propagated to high titers in Vero cells cultured in media supplemented with fetal bovine serum (FBS) (Monath et al., Biologicals 33:131-144, 2005). It was successfully tested in preclinical and Phase I and II clinical trials (Monath et al., Vaccine 20:1004-1018, 2002; Monath et al., J. Infect. Dis. 188:1213-1230, 2003). Similarly, successful Phase I clinical trials have been conducted with a ChimeriVax™-WN vaccine candidate, which contains the prM-E sequence from a West Nile virus (NY99 strain), with three specific amino acid changes incorporated into the E protein to increase attenuation (Arroyo et al., J. Virol. 78:12497-12507, 2004).

Summary of the Invention

The ~~invention~~present disclosure provides recombinant Flaviviruses that include one or more membrane (M) protein mutations (e.g., substitutions, deletions, or insertions), such as mutations that attenuate the Flavivirus (e.g., mutations that decrease the viscerotropism/viremia of the Flavivirus), increase genetic stability of the Flavivirus during propagation in cell culture (e.g., manufacturing in serum free cultures), and/or increase vaccine virus yields. The Flaviviruses of the ~~invention~~present disclosure can be chimeric Flaviviruses, such as Flaviviruses that include capsid and non-structural proteins of a first Flavivirus (e.g., a yellow fever virus, such as YF 17D) and membrane and/or envelope proteins of a second Flavivirus (e.g., Japanese encephalitis virus, West Nile

virus, a dengue virus (dengue-1, dengue-2, dengue-3, or dengue-4 virus), St. Louis encephalitis virus, Murray Valley encephalitis virus, tick-borne encephalitis virus, as well as any other Flavivirus that is a human/animal pathogen from the YF, JE, DEN, and TBE serocomplexes).

In the Flaviviruses of the ~~invention~~present disclosure, the mutation (e.g., substitution) can be in the transmembrane or ectodomain of membrane protein M. For example, the mutation can be in the region of amino acids 40-75 of the predicted membrane helix of the membrane protein M of the Flavivirus. As an example, the mutation can be a substitution of amino acid 60 of the membrane protein of a Flavivirus such as Japanese encephalitis virus (e.g., arginine to cysteine in the Japanese encephalitis virus M protein), or in a corresponding amino acid of another Flavivirus. Determination of which amino acid in a given Flavivirus "corresponds" to that of another Flavivirus can be carried out by standard amino acid sequence alignment, as is well known to those of skill in this art. As another example, the mutation can be a substitution of amino acid 66 of the membrane protein of a Flavivirus such as West Nile virus (e.g., a substitution of leucine with proline in the M protein of West Nile virus), or in a corresponding amino acid of another Flavivirus. In other examples, the mutation is at another membrane anchor amino acid, e.g., one or more amino acids selected from the group flanking the M66 residue, including positions 60, 61, 62, 63, 64, 65, and 66 of Japanese encephalitis virus or West Nile virus (or corresponding amino acids in other Flaviviruses) or other amino acid residues of the transmembrane domain.

We also provide for the first time evidence that the ectodomain of the M protein is of important functional significance, because a glutamine to proline change at the M5 residue increased the pH threshold of infection. Therefore, it can now be expected that Flavivirus attenuation can be achieved through amino acid changes or introduction of various deletions or insertions in the amino-terminal ectodomain, or surface part of the M protein, not only its C-terminal hydrophobic anchor. Thus, in other examples, the viruses of the ~~invention~~present disclosure include one or more mutations in the M protein ectodomain (residues 1-40) as described herein. This result is quite unexpected, given the lack of any known function of the mature M protein of Flaviviruses.

In addition to the membrane protein mutations noted above, in the case of chimeric Flaviviruses that include membrane and envelope proteins of a West Nile virus, the viruses of the ~~invention~~present disclosure can include one or more envelope protein mutations in amino acids selected from the group consisting of amino acids 107, 138, 176, 177, 224, 264, 280, 316, and 440. In other Flaviviruses, the mutations can be present in amino acids that correspond to these amino acids. As a specific example, the Flavivirus can include a mutation corresponding to mutation(s) in West Nile M protein amino acid 66 and E protein mutations at amino acids corresponding to West Nile virus amino acids 107, 316, and 440. In addition to the mutations described above, the Flaviviruses of the ~~invention~~present disclosure can also include one or more mutations in the hydrophobic pocket of the hinge region of the envelope protein, as described elsewhere herein. Further mutations that can be included in the viruses of the ~~invention~~present disclosure are mutations in the 3'UTR, the capsid protein, or other envelope protein regions, as described further below.

In view of the above, in one aspect, there is provided an attenuated, chimeric flavivirus comprising a yellow fever virus in which the membrane and envelope proteins of the yellow fever virus have been replaced with the membrane and envelope proteins of a Japanese encephalitis virus, wherein the transmembrane domain of the membrane protein of the chimeric flavivirus comprises a substitution of amino acid 60 of the Japanese encephalitis virus membrane protein.

In another aspect, there is provided an attenuated, chimeric flavivirus comprising a yellow fever virus in which the membrane and envelope proteins of the yellow fever virus have been replaced with the membrane and envelope proteins of a West Nile virus, wherein the transmembrane domain of the membrane protein of the chimeric flavivirus comprises a substitution of amino acid 66 of the West Nile virus membrane protein.

The ~~invention~~present disclosure also provides vaccine compositions that include the Flaviviruses described above and elsewhere herein and pharmaceutically acceptable carriers or diluents, as well as methods of inducing immune responses to Flaviviruses in patients by administration of such vaccine compositions. The patients treated according to such methods include those that do not have, but are at risk of developing, infection by the Flavivirus, as well as patients that are infected by the Flavivirus. Further, the ~~invention~~present disclosure includes the use of the Flaviviruses described herein in the prophylactic and therapeutic methods described herein, as well as in the manufacture of medicaments for these purposes.

In another aspect, there is provided a vaccine composition comprising the attenuated chimeric flavivirus as defined above and a pharmaceutically acceptable carrier or diluent.

In another aspect, there is provided the use of the vaccine composition as defined above in the manufacture of a medicament for inducing an immune response to Japanese encephalitis virus in a patient.

In another aspect, there is provided the use of the attenuated chimeric flavivirus as defined above to formulate a vaccine for inducing an immune response to Japanese encephalitis virus in a patient.

In yet another aspect, there is provided the use of the vaccine composition as defined above for in the manufacture of a medicament for inducing an immune response to West Nile virus in a patient.

In yet another aspect, there is provided the use of the attenuated chimeric flavivirus as defined above to formulate a vaccine for inducing an immune response to West Nile virus in a patient.

The ~~invention~~present disclosure further provides methods of producing vaccines that include a Flavivirus as described herein, which involve introducing into the membrane protein of the Flavivirus a mutation that results in decreased viscerotropism/viremia, and/or increased genetic stability/yields. Further, the ~~invention~~present disclosure provides nucleic acid molecules (RNA or DNA) corresponding to the genomes of the Flaviviruses described herein (or the complements thereof), and methods of using such nucleic acid molecules to make the viruses of the ~~invention~~present disclosure.

In yet another aspect, there is provided a method of producing a vaccine against Japanese encephalitis virus comprising an attenuated chimeric flavivirus, the method comprising introducing a mutation into the transmembrane domain of the membrane protein of a chimeric flavivirus comprising a yellow fever virus in which the membrane and envelope proteins of the yellow fever virus have been replaced with the membrane and envelope proteins of a Japanese encephalitis virus, wherein the mutation is a substitution of amino acid 60 of the membrane protein of said Japanese encephalitis virus, and wherein the mutation attenuates the chimeric flavivirus.

In yet another aspect, there is provided a method of producing a vaccine against West Nile virus comprising an attenuated chimeric flavivirus, the method comprising introducing a mutation into the transmembrane domain of the membrane protein of a chimeric flavivirus comprising a yellow fever virus in which the membrane and envelope proteins of the yellow fever virus have been replaced with the membrane and envelope proteins of a West Nile virus, wherein the mutation is a substitution of amino acid 66 of the membrane protein of said West Nile virus, and wherein the mutation attenuates the chimeric flavivirus.

In yet another aspect, there is provided a nucleic acid molecule corresponding to the genome of the attenuated chimeric flavivirus as defined above or the complement thereof.

In yet another aspect, there is provided a method of manufacturing the attenuated chimeric flavivirus as defined above, the method comprising introducing a nucleic acid molecule corresponding to the genome of the chimeric flavivirus into cells and isolating chimeric flavivirus produced in the cells from the cells or the supernatant thereof.

The Flaviviruses of the ~~invention~~present disclosure are advantageous because, in having decreased virulence (shown, e.g., by decreased viscerotropism/viremia), they provide an additional level of safety, as compared to their non-mutated counterparts, when

administered to patients. An additional advantage is that some mutations, such as the M-60 mutation in ChimeriVax™-JE, preclude accumulation of undesirable mutations during vaccine manufacture that otherwise could compromise safety, and increase manufacturing yields. Additional advantages of these viruses are provided by the fact that they can include sequences of yellow fever virus strain YF17D (e.g., sequences encoding capsid and non-structural proteins), which (i) has had its safety established for >60 years, during which over 350 million doses have been administered to humans, (ii) induces a long duration of immunity after a single dose, and (iii) induces immunity rapidly, within a few days of inoculation. In addition, the vaccine viruses of the ~~invention~~present disclosure cause an active infection in the treated patients. As the cytokine milieu and innate immune response of immunized individuals are similar to those in natural infection, the antigenic mass expands in the host, properly folded conformational epitopes are processed efficiently, the adaptive immune response is robust, and memory is established.

The beneficial aspects of mutations in the M protein on vaccine safety and manufacture in cell culture are novel and unexpected, given the lack of any known function of the mature M protein of Flaviviruses.

Other features and advantages of the ~~invention~~present disclosure will be apparent from the following detailed description, the drawings, and the claims.

Brief Description of the Drawings

Fig. 1A is a schematic representation of the 3' untranslated region of yellow fever virus, which shows domains within this region (repeat sequences (RS), conserved sequences CS2, CS1, and the 3'-extreme stem-loop structure), as well as examples of mutations that can be included in the viruses of the invention (e.g., deletions dA, dB, dC, dD, d7, d14, CS2 d5, and CS2 d16).

Fig. 1B is a schematic representation of the sequence and published secondary structure prediction of the 3' untranslated region of yellow fever 17D virus, from the middle of the 3rd RS element to the end of the UTR (Proutski et al., J. Gen. Virol. 78:1543-1549, 1999) (SEQ ID NO: 31).

Fig. 1C is an illustration of the optimal YF 17D 3'UTR secondary structure prediction produced using the Zuker RNA folding algorithm (SEQ ID NO: 32).