Description

Title of Invention: FLAVIVIRUS VIRUS LIKE PARTICLE

Technical Field

[0001] The present application relates to a virus like particle comprising one or more flavivirus structural proteins, and a composition or vaccine comprising thereof, its use in medicine, particularly in the prevention or treatment of flavivirus infection.

Background Art

[0002] Flavivirus comprise more than 70 different viruses, many of which are arthropod-borne and transmitted by either mosquitoes or ticks.

[0003] Flavivirus is a genus of viruses in the family Flaviviridae. This genus includes the West Nile virus (WNV), dengue virus (DENV), tick-borne encephalitis virus (TBEV), yellow fever virus (YFV), Japanese encephalitis (JEV), hepatitis C virus (HCV) and several other viruses which may cause encephalitis or haemorrhagic diseases.

[0004] Dengue fever is a mosquito-borne disease caused by the flavivirus and has spread to most tropical and many subtropical areas. The disease is caused by four closely related viruses, the Dengue virus 1 including subtypes I to IV, Dengue virus 2 including subtypes Asian I, Asian II, Cosmopolitan, American and American/Asian, Dengue virus 3 including subtypes I to IV and Dengue virus 4 including subtypes I to III. Although Dengue is the most important flavivirus with respect to global disease incidence, the development and use of vaccines against the virus has been hampered so far by the theoretical risk of vaccine-related adverse events such as immune enhancement of infection and the requirement to induce a long-lasting protective immune response against all four dengue serotypes simultaneously.

[0005] There is no effective dengue therapeutic and prevention against dengue fever is currently limited to vector control measures. A dengue vaccine would therefore represent a major advance in the control of the disease.

[0006] While no licensed dengue vaccine is available, several vaccine candidates are currently evaluated in clinical studies. WHO indicates that the growing global epidemic of dengue is of mounting concern, and a safe and effective vaccine is urgently needed.

(http://www.who.int/immunization/research/development/dengue_vaccines/en/) and Vaccine 30 (2012) 4301-4306)

[0007] Virus-like particles (VLPs) are multiprotein structures that mimic the organization and conformation of authentic native viruses but non-infectious because they do not contain any viral genome, potentially yielding safer vaccine candidates. A handful of prophylactic VLP-based vaccines is currently commercialized worldwide: Glaxo-

SmithKline's Engerix[R] (hepatitis B virus) and Cervarix[R] (human papillomavirus), and Merck and Co., Inc.'s Recombivax HB[R] (hepatitis B virus) and Gardasil[R] (human papillomavirus) are some examples. Other VLP-based vaccine candidates are in clinical trials or undergoing preclinical evaluation, such as, influenza virus, parvovirus, Norwalk and various chimeric VLPs. Many others are still restricted to small-scale fundamental research, despite their success in preclinical tests. The implications of large-scale VLP production are discussed in the context of process control, monitorization and optimization. The main up- and down-stream technical challenges are identified and discussed accordingly. Successful VLP-based vaccine blockbusters are briefly presented concomitantly with the latest results from clinical trials and the recent developments in chimeric VLP-based technology for either therapeutic or prophylactic vaccination.

[0008] Up to now, VLP-based vaccines have been produced for more than 30 different viruses that infect human and other animals. The examples include AAV (Adeno-associated virus), H5N3 (Avian influenza), BFDV (Budgerigar fledgling disease virus), BTV (Bluetongue virus), Ebola, Enterovirus 71, GHPV (Goose hemorrhagic polyoma virus), HBV (Hepatitis B virus), HCV (Hepatitis C virus), HDV (Hepatitis δ virus), HEV (Hepatitis E virus), HIV, HPV (Human papillomavirus), IBDV (Infectious bursal disease virus), Influenza A, Influenza A H1N1, Influenza A H3N2, JC polymavirus, Margurg, MS2, IPCV (Indian peanut clump virus), NDV (Newcastle disease virus), No (Norovirus) Nv (Norwalk virus), PhMV (Physalis mottle virus), Polymavirus, PPV (Porcine parvovirus), RHDV (Rabbit hemorrhagic disease virus), Rotavirus, SARS, SIV (Simian immunodeficiency virus), SV40 (Simian virus 40), SVDV (Swine vesicular disease virus) and so on. (Expert Rev. Vaccines 9(10), 1149-1176, 2010).

Citation List

Non Patent Literature

[0009] [NPTL1]Vaccine 30 (2012) 4301-4306

[NPTL2] Expert Rev. Vaccines 9(10), 1149-1176, 2010

SUMMARY OF THE INVENTION

[0010] In a first aspect, the present application provides a virus like particle comprising one or more flavivirus structural proteins, wherein the envelope region of the flavivirus structural protein contains at least one alteration in the amino acid sequence.

[0011] In a second aspect, the present application provides a nucleic acid molecule comprising or consisting of a nucleotide sequence that encodes the flavivirus structural protein contained in the virus like particle provided in the first aspect of the present application.

[0012] In a third aspect, the present application provides a composition or vaccine comprising the virus like particle provided in the first aspect and/or the nucleic acid molecule provided in the second aspect.

[0013] In a fourth aspect, the present application provides a method of producing an antibody or anti-serum comprising a neutralizing antibody, comprising contacting the virus like particle provided in the first aspect of the present application and/or the nucleic acid molecule provided in the second aspect of the present application to a mammal.

[0014] In a fifth aspect, the present application provides a method of treating or preventing flavivirus infection or a method of inducing and/or enhancing immune response against a flavivirus in a mammal, comprising administering the composition provided in the third aspect of the present application to the mammal.

[0015] In a sixth aspect, the present application provides a method of producing the virus like particle provided in the first aspect of the present application, comprising: culturing a cell which is transfected with a gene encoding the at least one flavivirus structural proteins contained in the virus like particle; and recovering virus like particle from the cell culture. This aspect may further comprise the step of preparing a gene comprising a nucleotide sequence encoding the at least one flavivirus structural proteins contained in the virus like particle.

[0016] In a seventh aspect, the present application provides a virus like particle for use in a method or kit of diagnosing flavivirus infection in a mammal.

Brief Description of Drawings

[0017] [fig.1]Figure 1 shows the construction of Dengue virus genome and one example of dengue VLP construct.

[fig.2]Figure 2 shows envelope proteins in the supernatants measured by Western Blotting. Expression vector for viral structural protein comprising Dengue type 2 virus prM and envelope protein, or for a viral structural protein comprising Dengue type 2 virus prM and a chimera of Dengue type 2 and Dengue type 1, wherein the C-terminal region of the Dengue virus type 2 envelope protein was replaced to the corresponding Dengue virus type 1 C-terminal region (199aa-676aa of SEQ ID NO: 8), or a viral structural protein containing the chimeric envelope region wherein one amino acid alteration in the chimeric envelope region (F108K, at 289aa of SEQ ID NO: 10) was introduced, and an expression vector for Dengue virus type 1 capsid protein were transfected to 293F cells. Western blotting using antibody against Dengue Envelope protein was conducted against the culture supernatant.

[fig.3]Figure 3 shows results of Western Blotting. An expression vector for viral structural protein comprising Dengue type 1 virus prM and envelope with or without

alteration (wild type, F108A, K203M, K246M F108A +K246M or F108+K203N) together with expression vector for Dengue type 1 Capsid protein were transfected to 293F cells. Western blotting using antibody against Dengue Envelope protein was conducted against the culture supernatant.

[fig.4]Figure 4 shows results of Western Blotting. An expression vector for viral structural protein comprising Dengue type 1 virus prM and envelope with or without alteration (wild type, F108A, K203M or F108+K203N) was transfected to 293F cells. Western blotting using antibody against Dengue Envelope protein was conducted against the culture supernatant.

[fig.5]Figure 5 shows the Electron microscopic (EM) visualization of negatively stained VLPs (F108+K203N).

Description of Embodiments

[0018] (1) A virus like particle comprising one or more flavivirus structural proteins

In the first aspect, the present application provides a virus like particle comprising one or more flavivirus structural proteins, wherein at least one amino acid in the envelope region is altered from its naturally occurred structure.

[0019] Flavivirus may be West Nile virus, Dengue virus, tick-borne encephalitis virus, yellow fever virus, Japanese encephalitis, and hepatitis C virus (HCV). Preferred flavivirus is Dengue virus. Dengue virus may be Dengue virus 1 including subtypes I to IV, Dengue virus 2 including subtypes Asian I, Asian II, Cosmopolitan, American and American/Asian, Dengue virus 3 including subtypes I to IV and Dengue virus 4 including subtypes I to III. In the specification and claims, the term "flavivirus structural protein or fragment thereof", refers any peptide-based sequence derived from flavivirus that can be recognized by the immune system in a subject, and/or that stimulates a cell-mediated immune response in a subject and/or stimulates the generation of antibodies in a subject.

[0020] As shown in Figure 1, Dengue virus structural protein consists of capsid protein, precursor membrane protein and envelope protein. In this embodiment, VLP comprises at least one of those structural proteins and preferably, a precursor membrane protein (prM) and an envelope protein. The Dengue virus structural protein may further comprise an amino acid corresponding to the initiation codon and a signal sequence to the amino terminal of the prM sequence.

[0021] The region comprising at least one amino acid alternation may be preferably between amino acid position 182 and amino acid position 676, more preferably between amino acid position 271 and amino acid position 302, especially between amino acid position 280 and amino acid position 291, particularly 289 of a protein of SEQ ID NO: 21 which comprises initiation codon M and signal peptide of SEQ ID NO: 17 followed by

prM and envelope region of Dengue Virus 1 (WestPac strain, GenBank Accsession No: U88535), or between the positions determined as the above-identified positions when the amino acid sequence of a protein comprising initiation codon M, signal peptide followed by prM and envelope region of a flavivirus or a fragment thereof is aligned with the amino acid sequence represented by SEQ ID NO: 21.

[0022] The envelope region of the dengue virus type 1 amino acid sequence is represented by SEQ ID NO: 20. Accordingly, the region comprising at least one amino acid alternation may be in this sequence, preferably between amino acid position 90 and amino acid position 121, especially between amino acid position 99 and amino acid position 110, particularly 108 of a protein of SEQ ID NO: 20, or between the positions determined as the above-identified positions when the amino acid sequence of an envelope of a flavivirus or a fragment thereof is aligned with SEQ ID NO: 20.

[0023] As used herein, "a position corresponding to" or recitation that nucleotides or amino acid positions "correspond to" nucleotides or amino acid positions in a disclosed sequence, such as set forth in the Sequence listing, refers to nucleotides or amino acid positions identified upon alignment with the disclosed sequence to maximize identity using a standard alignment algorithm. By aligning the sequences, one skilled in the art can identify corresponding residues.

[0024] Viral structural proteins of various flavivirus such as dengue virus types 1-4 have been identified and available at various public databases such as GenBank database. For example, Dengue virus type 1 (WestPac strain): Accession No. U88535, Dengue virus type 2 (S1 vaccine strain): Accession No. M19197, Dengue virus type 3 (strain Singapore 8120/95): Accession No. AY766104 and Dengue virus type 4 (strain ThD4_0476_97): Accession No. Y618988. According to this application, envelope protein of a flavivirus such as dengue virus may be obtained from a database and aligned with SEQ ID NO: 20 to identify a position between the positions corresponding to amino acid position 90 and amino acid position 121 of SEQ ID NO: 20, such as a position corresponding to at amino acid position 108 of SEQ ID NO: 20.

[0025] With the exception of comprising at least one amino acid alteration in the envelope region, a flavivirus structural protein contained in the virus like particle may be a naturally occurring viral structural protein or a modified protein thereof. In one embodiment, the modified protein has at least 70%, 75%, 80%, 85%, 90%, 95% or 98% amino acid sequence identity to a naturally occurring viral structural protein including prM and envelope protein. In one embodiment, the modified protein is a mutant where at most 10% of the amino acids are deleted, substituted, and/or added to a naturally occurring viral structural protein including prM and envelope regions. The sequence identity may be determined by conventional methods.

[0026] According to the present application, one or more flavivirus structural proteins or

fragments thereof discussed above may be used as long as they spontaneously assemble into a particulate structure. For example, when eukaryotic cells expressing a gene encoding prM and envelope proteins of dengue virus are cultured, the proteins are generated by the cells and assemble to give VLPs, and the VLPs can be collected from the cell culture supernatant.

[0027] The present application addresses one or more of the above needs by providing VLPs, vectors encoding the VLPs, and antibodies (and antibody-like molecules including aptamers and peptides) that specifically bind to the antigen, together with the uses thereof (either alone or in combination) in the prevention or treatment of flavivirus infections.

[0028] As used in the specification and claims, the term "antibody" refers to a molecule which is capable of binding to an epitope or antigenic determinant. The term covers a whole antibody and an antigen-binding fragment thereof, including a single-chain antibody. Such antibodies may include human antigen binding antibody fragments and include, but are not limited to, Fab, Fab' and F(ab')2, Fd, single-chain Fvs (scFv), single-chain antibodies, disulfide-linked Fvs (sdFv) and fragments comprising either a VL or VH domain. The antibodies can be from any animal origin including birds and mammals. Preferably, the antibodies are mammalian e.g. human, murine, rabbit, goat, guinea pig, camel, horse and the like, or other suitable animals e.g. chicken. As used herein, "human" antibodies include antibodies having the amino acid sequence of a human immunoglobulin and include antibodies isolated from human immunoglobulin libraries or from animals transgenic for one or more human immunoglobulins and that do not express endogenous immunoglobulins, as described, for example, in U.S. Patent No. 5,939,598, the disclosure of which is incorporated herein by reference in its entirety.

[0029] A flavivirus structural protein may be a naturally occurring protein or modified protein of the naturally occurring protein or a fragment of the naturally occurring protein or the modified peptide. The modified protein may be a fragment of the naturally occurring virus structural protein.

[0030] In one embodiment, the modified protein derived from a flavivirus structural protein has at least 70%, 75%, 80%, 85%, 90%, 95% or 98% amino acid sequence identity to the naturally occurring protein. In one embodiment, the modified protein derived from a flavivirus is a mutant where at most 10% of the amino acids are deleted, substituted, and/or added based on the naturally occurring viral structural protein derived from the flavivirus.

[0031] In one embodiment, the present application provides a virus like particle comprising a flavivirus structural protein having an amino acid sequence represented by any one of SEQ ID Nos.2, 4, 8, 10, 14 and 16.

[0032] Each of those sequences represents a protein containing the following regions: initiation codon: M (1aa), signal sequence (2-15aa), pr sequence (16-106aa), M sequence (107-181aa), and Envelope region (182-676aa). In one embodiment, a virus like particle comprising a viral structural protein having the regions of prM and envelope region of SEQ ID Nos.2, 4, 8, 10, 14 and 16, i.e. comprising amino acid sequences of from position 16 to position 676 of those proteins may be provided.

[0033] The modified flavivirus structural protein may have at least 70%, 75%, 80%, 85%, 90%, 95% or 98% amino acid sequence identity to an amino acid sequence represented by any one of SEQ ID Nos.2, 4, 8, 10, 14 and 16, or an amino acid sequence from position 16 to position 676 of the one of those sequences. Also, the modified flavivirus structural protein may be a mutant where at most 10% of the amino acids are deleted, substituted, and/or added based on the flavivirus structural protein having an amino acid sequence represented by any one of SEQ ID Nos. SEQ ID Nos.2, 4, 8, 10, 14 and 16, or an amino acid sequence from position 16 to position 676 of any one of those sequences.

[0034] (2) Nucleotide and Vector

In the second aspect, the present application provides a nucleic acid molecule comprising or consisting of a nucleotide sequence encoding the virus like particle as provided in the first aspect of the present invention.

[0035] In one embodiment, the present application provides a nucleic acid molecule comprising a nucleotide sequence that encodes a flavivirus structural protein that provide the virus like particle as described above.

[0036] In one embodiment, the present application provides an expression vector comprising the nucleic acid molecule as described above, wherein the vector optionally comprises an expression control sequence operably linked to the nucleic acid molecule.

[0037] Examples of expression control sequences include, but are not limited to, promoter such as CMV promoter, phage lambda PL promoter, the E. coli lac, phoA and tac promoters, the SV40 early and late promoters, and promoters of retroviral LTRs.

[0038] In one embodiment, the present application provides an expression vector for a flavivirus structural protein, which consists of a nucleotide sequence represented by any one of SEQ ID Nos.1, 3, 7, 9, 13 and 15.

[0039] In one embodiment, the present application provides a nucleic acid molecule which is modified from the nucleic acid molecule having a nucleotide sequence represented by any one of SEQ ID Nos.1, 3, 7, 9, 13 and 15. The modified nucleic acid molecule may have at least 70%, 75%, 80%, 85%, 90%, 95% or 98% nucleotide sequence identity to the nucleic acid molecule having a nucleotide sequence represented by any one of SEQ ID Nos.1, 3, 7, 9, 13 and 15. Also, the modified nucleic acid molecule may be a mutant where at most 10% of the amino acids are deleted, substituted, and/or

added based on the nucleic acid molecule having a nucleotide sequence represented by any one of SEQ ID Nos.1, 3, 7, 9, 13 and 15.

[0040] (3) Composition or vaccine

In the third aspect, the present application provides a composition or vaccine comprising the virus like particle provided in the first aspect of the present application and/or the nucleic acid molecule provided in the second aspect of the present invention.

[0041] In one embodiment, the present application provides a composition comprising the flavivirus virus like particle as described above or the nucleic acid molecule as described above.

[0042] The composition may further comprise a pharmaceutically acceptable carrier and/or adjuvant. Examples of adjuvant include, but are not limited to Ribi solution (Sigma Adjuvant system, Sigma-Aldrich).

[0043] The pharmaceutical composition of the present application may contain a single active ingredient or a combination of two or more active ingredients, as far as they are not contrary to the objects of the present invention. For example, cytokines including chemokines, antibodies against a cytokine such as anti-TNF antibody (e.g. infliximab, adalimumab), anti-VEGF antibody (e.g. bevacizumab and ranibizumab), cytokine receptor antagonist such as anti HER2 antibody (e.g. Trastuzumab), anti EGF receptor antibody (e.g. Cetuximab), anti VEGF aptamer (e.g. Pegaptanib) and immunomodulator such as cyclosporine, tacrolimus and ubenimex may be used for the combination therapy.

[0044] In a combination of plural active ingredients, contents of the respective ingredients may be suitably increased or decreased in consideration of their therapeutic effects and safety.

[0045] The term "combination" used herein means two or more active ingredients are administered to a patient simultaneously in the form of a single entity or dosage, or those active ingredients are administered to a patient as separate entities either simultaneously or sequentially with no specific time limits, wherein such administration provides therapeutically effective levels of the two components in the body, preferably at the same time.

[0046] In one embodiment, the composition is a vaccine composition including a DNA vaccine. In one embodiment, the DNA vaccine provided by the present invention comprises CpG containing oligonucleotide.

[0047] (4) Method of producing an antibody

In the fourth aspect, the present application provides a method of producing an antibody against a flavivirus or an antiserum containing a neutralizing antibody against a flavivirus, comprising contacting the virus like particle provided in the first aspect of

the present application and/or the nucleic acid molecule provided in the second aspect of the present invention to a mammal.

[0048] The antibody produced in this aspect may be used for passive immunization against a flavivirus-causing pathogen in a mammal by administering the same to the mammal, and thus prevent the mammal from flavivirus infection or treat a disease or condition caused by flavivirus infection in the mammal.

[0049] The antibody produced in the fourth aspect of the present application may be humanized using a conventional technique. Thus, in one embodiment, the method provided in the fourth aspect of the invention further comprises a step of humanizing a non-human mammal produced antibody. The antibody or humanized antibody provided by this aspect may be used for preventing a human subject from flavivirus infection or for treating a disease or condition caused by flavivirus infection in the subject.

[0050] The antibody produced according to this aspect may be used in vitro to select a sub-population from immune cells such as B-cell and T-cell derived from the patient, which are then re-administered to the patient.

[0051] Antiserum can be obtained by the conventional manner. Blood samples are taken from the immunized non-human animal, and the blood is processed so as to obtain the antiserum, i.e. the antibody-containing liquid component of the blood. The non-human mammal is preferably selected from the group consisting of rat, mouse, hamster, pig, rabbit, horse, donkey, goat, sheep, guinea pig, lama, and non-human primate (e.g. chimp).

[0052] (5) Method of treating a disease caused by a flavivirus infection in a subject or preventing a subject from flavivirus infection

In the fifth aspect, a method of treating a disease or condition caused by flavivirus infection such as dengue fever in a subject, wherein the virus like particle provided in the first aspect, the nucleotide molecule provided in the second aspect, or the composition provided in the third aspect is administered to the subject. By administering the above listed VLP, nucleotide molecule or composition to the subject, immune response against a flavivirus can be enhanced and thus, the disease or condition caused by the flavivirus infection can effectively be treated. In this aspect, the VLP, nucleotide molecule or composition may be administered to the patient locally to the affected organ or systemically.

[0053] A method of preventing a subject from a flavivirus infection or from a disease caused by a flavivirus infection, comprising administering the virus like particle of the first aspect, the nucleotide molecule provided in the second aspect or the composition of the third aspect to the subject. The disease caused by a flavivirus may be dengue fever.

[0054] According to the present application, the virus like particle can also be applied for

immune therapy. The VLP may be applied ex vivo to cells derived from the patient or a human cell line which are subsequently administered to the patient.

[0055] (6) Method of producing the virus like particle

In the sixth aspect, the present application provides a method of producing the virus like particle provided in the first aspect of the present invention, comprising culturing a cell which is expressing a gene coding for the viral structural protein; and recovering the virus like particle from the cell culture.

[0056] Various host-vector systems may be used for expression of the virus like particle. Eukaryotic cells can be used for the method provided by the fourth aspect of the present application. Examples of eukaryotic cells include, but are not limited to, insect cells (e.g. sf9 cells, H5 cells), yeast cells (e.g. S. cerevisiae) and mammalian cells (e.g. CHO cells, human embryonic kidney (HEK) 293F cells). Vector used for the method provided by the second aspect of the present application comprises a nucleic acid molecule encoding the virus like particle to be expressed. Cells may be transfected with the vector using conventional methods (e.g. lipofection, electroporation). A skilled person can select culture medium or with DNA methyl transferase inhibitors and histone deacetylase inhibitors such as sodium butyrate, depending on cells employed. After the transfection, virus like particle can be produced in the cells and/or culture supernatant. Virus like particle may be recovered from the culture supernatant and purified using ultracentrifugation.

[0057] The virus like particles of the present application do not replicate and therefore, have highly safe profiles.

[0058] (7) Kit or method of diagnosing flavivirus infection

In the seventh aspect, the present application provides a virus like particle for use in a method or kit of diagnosing flavivirus infection in a mammal. By using the virus like particles, an enzyme-linked immune-sorbent assay (ELISA) diagnostic kit capable of detecting an antibody specific to the flavivirus could be produced.

[0059] The present application will be described in detail with reference to the following example, which, however, is not intended to limit the scope of the present invention.

Example 1

[0060] Preparation of a virus like particle comprising a protein or a fragment of Dengue virus type 1-2

Dengue virus types 1 and 2 viral structural proteins were used as the wild type viral structural proteins. In the envelope region of the viral structural proteins, at least one alternation was introduced. Namely, amino acid Phe at position 108 of Dengue virus type 1 envelope protein (SEQ ID NO: 20) was altered to Ala (F108A), amino acid Lys at position 203 of Dengue virus type 1 envelope protein was altered to Asn (K203N)

(Envelope region: 172-676aa of SEQ ID NO: 4), amino acid Asn at position 246 was altered to Met (K246M). In this example, envelope region corresponds to 172-676aa of SEQ ID NO: 2, position 108 of SEQ ID NO: 20 corresponds to position 289 of SEQ ID NO: 2, position 203 of SEQ ID NO: 20 corresponds position 384 of SEQ ID NO: 2, and position 246 of SEQ ID NO: 20 corresponds to position 427 of SEQ ID NO: 2. In the examples, mutation in the envelope region is expressed using the number starting from the envelope region.

[0061] To express the viral structural protein in mammalian cells, DENV2 prM E expression vector and DENV2 Capsid expression vector 20ug each were mixed and transfected to 293F cells. Capsid vector was mutated R85A and K86A to express better yield (SEQ ID NO: 11 and 12). To express the DENV2 prM E better, C terminal DENV2 Env region (199aa-676aa) was replaced to the corresponding DENV1 Env region to give chimera proteins(SEQ ID NOs. 7 and 8). To stabilize the VLPs, Env fusion peptide region (F108A) was mutated (SEQ ID NOs. 9 and 10).

[0062] 4 days after the transfection, supernatant from the transfected cell culture was harvested and the Env expression was examined by Western blotting using Dengue virus (9.F.10) monoclonal antibody that specifically binds to the envelope protein (sc-70959, Santa Cruz Biotechnology) as a 1st antibody and rabbit anti-mouse IgG-HRP (sc-358920, Santa Cruz Biotechnology)as a 2nd antibody. The result is shown in Fig. 2.

[0063] As shown from Figure 2, cells transfected with DENV2 prM and the chimera of Dengue virus type 2 and type 1 envelope protein with one alteration (F108A) generated higher amount of protein. Those proteins in the supernatant spontaneously assembled to give particles.

[0064] To express the VLP, DENV1 prM E expression vector and DENV1 Capsid expression vector 20ug each were mixed and transfected to 293F cells. Capsid vector was mutated K85A and K86A to express better yield (SEQ ID NOs. 5 and 6). To stabilize the VLPs, Env fusion peptide region (F108A) (SEQ ID NOs. 1 and 2) or basic amino acid (K203N) (SEQ ID NOs. 3 and 4) or (K246M) were mutated. Further, two amino acids (F108A+K246M, SEQ ID NOs: 13 and 14) and (F108A+K203N, SEQ ID NOs. 15 and 16) were also mutated. 4 days after transfection, supernatant from the transfected cells was harvested and the Env expression was examined by Western blotting using Dengue virus (9.F.10) monoclonal antibody (sc-70959, Santa Cruz Biotechnology) as a 1st antibody and rabbit anti-mouse IgG-HRP (sc-358920, Santa Cruz Biotechnology)as a 2nd antibody. Results are shown in Fig. 3.

[0065] As shown by Figure 3, cells transfected with DENV prM and the envelope protein with at least one alteration generated higher amount of envelope protein. Those proteins in the supernatant spontaneously assembled to give particles.

Example 2

[0066] Preparation of a virus like particle comprising Dengue virus structural protein prM and envelope.

Expression vector for Dengue virus structural proteins containing prM and modified Envelope protein F108A, K203N, or K203N+F108A (SEQ ID NOs 1, 3, 15 respectively) used in Example 1 were used. In the same manner as Example 1, 20ug of the vector was transfected to 293F cells and cultured. Supernatants from the transfected cells were harvested on day 4 after the transfection. Western Blot was performed to detect DENV VLP using a monoclonal antibody (9.F. 10, Santa Cruz Biotech) as a primary antibody and rabbit anti-mouse IgG-HRP conjugated antibodies as a secondary antibody. Results are shown in Figure 4. As shown by Figure 4, cells transfected with DENV1-prM and the envelope protein with at least one alteration generated higher amount of envelope protein.

[0067] The supernatant was filtrated using 0.45μm filter to obtain virus like particles. The virus like particles were concentrated using TFF column and purified using QXL column (GE Healthcare) to obtain purified virus like particles. The purified VLP was filed in 4% formaldehyde in PBS. Electron microscopic (EM) visualization of negatively stained VLPs was performed. Briefly, 1.0μl of the sample was placed onto a carbon coated Formvar-filmed copper grid and allowed the VLPs to attach. Then, 2μl 1% PTA solution was added to the grid and the grid was examined by electron microscope. The result is shown in Figure 5. In the supernatant, virus like particles were observed.

Example 3

[0068] Antibody against Dengue virus

Purified VLP obtained in Example 2 (K203N+F108A) was named as DEN1 VLP and used in this example. The purified virus like particles were further concentrated using spin column (Molecular Weight-cutoff: 100kDa) to prepare the virus like particles for the immunization. Then, four (4) mice were immunized with DEN1 VLP 30 μg in PBS containing aluminum adjuvant (alhydrogel 2%, Sergeant Aduvants) by intramuscle injection two times at weeks 0 and 4.

[0069] Dengue virus specific IgG titers of the serum derived from the immunized mice were determined by ELISA system. The serum was assayed for neutralizing antibody to each of DENV-1, DENV-2, DENV-3, and DENV-4 virus. The following Dengue serotypes 1-4 were used:

DENV-1, Philippine-99St12A strain

DENV-2, Philippine-00St22A strain

DENV-3, Philipine-SLMC50 strain

DENV-4, Philippine-SLMC318 strain
JEV- JaOAr S-982 strain

[0070] Anti-DENV neutralizing antibodies in the immunized mice sera were detected by a previously described focus reduction neutralization test (FRNT) on Vero cells using 1.25 x 10^8 FFU of Vero-adapted DVs type 1-4. The endpoint titer was calculated as the highest serum dilution tested that reduced the number of FFU by at least 50% ($FRNT_{50}$). Vero cells at 100% confluence in 96 wells plates were used.

[0071] In house anti-Dengue rabbit IgG was used as the primary antibody, and anti-rabbit IgG-HRPO conjugate (102-PD) was used as the secondary antibody. The results are summarized in table below.

[0072] [Table 1]

serotype	D-1	D-2	D-3	D-4	JE
DENV1 VLP	>10240	1043	604	270	<80
PBS	<80	<80	<80	<80	<80

[0073] As shown in table 1, the DENV1 VLP exhibited superior neutralizing effect on Dengue virus types 1, 2, 3 and 4. In view of the fact that the FRNT 50 value of neutralizing antibodies obtained with Dengue attenuated live vaccine is around 1000 (Tsai et al., Journal of Virology, *2015 89:* 7348–7362.), the immunogenicity of DENV1 VLP was very strong and provided about 10 times higher neutralizing antibody against Dengue virus than attenuated live vaccine.

[EXAMPLE 4]

[0074] Preparation of a pharmaceutical composition comprising Dengue virus like particle

Dengue virus like particles were prepared according to Example 1. To prepare a pharmaceutical composition which is a vaccine composition, 80μg of each of the prepared particles was mixed with 1ml of Sucrose Phosphate Solution, pH 7.2, Endotoxin Free (Teknova, SP buffer).

[EXAMPLE 5]

[0075] Preparation of a Dengue virus Type 2-4 derived Virus Like Particles

Expression vectors for prM and Envelope proteins of Dengue virus type 2-4, wherein amino acid position 108 of the envelope region (corresponding to 289aa of SEQ ID NOs. 22-24) is replaced from F to A and C-terminal region of the Envelope protein (478-676aa or 476-674aa) is replaced with that corresponding to Dengue virus type 1 Envelope protein (478-676aa of SEQ ID NO. 22) are prepared. Proteins to be expressed by the vectors are those represented by SEQ ID NOs. 22-24. Virus like particles are obtained in the same manner as Example 1.

CLAIMS

1. A virus like particle comprising a flavivirus envelope protein, wherein said envelope protein comprises an amino acid substitution at position 108 of SEQ ID NO: 20, or at a position determined as corresponding to position 108 of SEQ ID NO: 20 by alignment.

2. The virus like particle according to claim 1, wherein the flavivirus is West Nile virus, dengue virus, tick-borne encephalitis virus, yellow fever virus, or Japanese encephalitis virus.

3. The virus like particle according to claim 2, wherein said flavivirus is dengue virus.

4. The virus like particle according to claim 1, wherein said virus like particle is produced from a dengue virus structural protein comprising prM and envelope regions.

5. The virus like particle according to claim 4, wherein said structural protein comprises the amino acid sequence of residues 16-676 of an amino acid sequence selected from the group consisting of SEQ ID NOs: 2, 4, 8, 10, 14 and 16.

6. The virus like particle according to claim 4, wherein said structural protein is a protein consisting of an amino acid sequence selected from the group consisting of SEQ ID NOs: 2, 4, 8, 10, 14 and 16.

7. A composition comprising the virus like particle according to claim 1 and a pharmaceutically acceptable carrier.

8. The composition according to claim 7 for treating or preventing a disease or condition caused by flavivirus infection.

9. Use of the composition according to claim 7 in the manufacture of a medicament for treating or preventing a disease or condition caused by flavivirus infection.

10. A method of producing a virus like particle, comprising culturing a cell which is transfected with a polynucleotide encoding the envelope protein contained in the virus like particle according to claim 1; and recovering the virus like particle from the cell culture.

11. The virus like particle according to claim 1, wherein said virus like particle further comprises a prM protein.

12. The virus like particle according to claim 1, wherein said envelope protein further comprises an amino acid substitution at position 203 of SEQ ID NO: 20, or at a position determined as corresponding to position 203 of SEQ ID NO: 20 by alignment.

13. The virus like particle according to claim 1, wherein said envelope protein further comprises an amino acid substitution at position 246 of SEQ ID NO: 20, or at a position determined as corresponding to position 246 of SEQ ID NO: 20 by alignment.

14. The virus like particle according to claim 1, wherein the substitution at position 108, or at the position corresponding to position 108, is the substitution of phenylalanine with alanine.

15. The virus like particle according to claim 12, wherein the substitution at position 203, or at the position corresponding to position 203, is the substitution of lysine with asparagine.

16. The virus like particle according to claim 13, wherein the substitution at position 246, or at the position corresponding to position 246, is the substitution of lysine with methionine.