(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
2 May 2013 (02.05.2013)

WIPO | PCT

(10) International Publication Number
WO 2013/063248 A1

(51) International Patent Classification:
C07K 19/00 (2006.01) *C07K 14/18* (2006.01)
A61K 39/12 (2006.01) *A61P 31/14* (2006.01)

(21) International Application Number:
PCT/US2012/061893

(22) International Filing Date:
25 October 2012 (25.10.2012)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/550,982 25 October 2011 (25.10.2011) US

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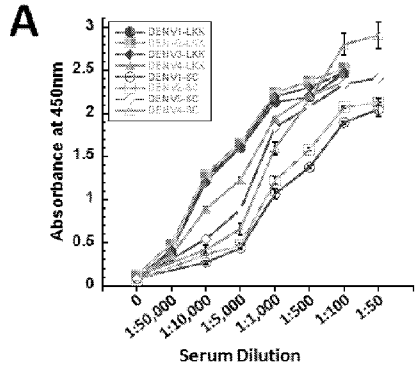
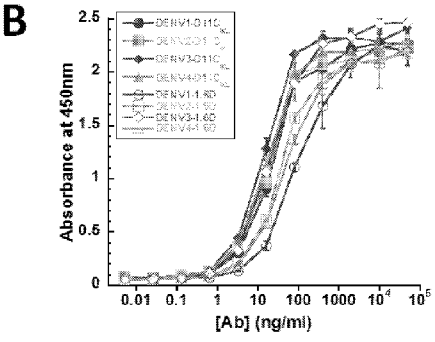
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ,

[Continued on next page]

(54) Title: VACCINES AND METHODS FOR CREATING A VACCINE FOR INDUCING IMMUNITY TO ALL DENGUE VIRUS SEROTYPES





(57) Abstract: Described here is a method to produce a chimeric protein having portions of yellow fever virus and dengue virus. A small portion of the yellow fever virus 17D vaccine strain envelope protein (or other related flavivirus) can be replaced by the corresponding portion from the dengue virus envelope protein. In some embodiments the chimeric protein may be used to create a treatment composition for DENV infection. In others, the chimeric protein may be used to create a vaccine that will induce broadly protective antibodies against dengue virus and reduce the induction of non-neutralizing antibodies that will cause enhancement.

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TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

— *with sequence listing part of description (Rule 5.2(a))*

VACCINES AND METHODS FOR CREATING A VACCINE FOR INDUCING IMMUNITY TO ALL DENGUE VIRUS SEROTYPES

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims priority to and the benefit of U. S. Provisional Patent Application No. 61/550,982, filed on October 25, 2011, and is incorporated herein by reference in its entirely.

GOVERNMENT SUPPORT

[0002] This invention was made with government support under cooperative agreements awarded by grant no. HDTRA1-10-1-0009 from the Defense Threat Reduction Agency. The government may have certain rights to the invention.

BACKGROUND OF THE INVENTION

[0003] Dengue viruses (DENV), members of the genus *Flavivirus*, are the most common cause of mosquito-borne viral diseases in tropical and subtropical regions around the world. Approximately 50 to 100 million people per year are infected with DENV. DENV infections may be asymptomatic, but most often manifest as dengue fever (DF), a self-limited disease. Dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) are more severe, life-threatening manifestations of dengue infection. DENV imposes one of the largest social and economic burdens of any mosquito-borne viral pathogen. There is no specific treatment for infection, and control of dengue virus by vaccination has proved elusive. The pathogenesis of DHF/DSS is not completely understood. There are four serotypes of dengue virus (DENV-1, DENV-2, DENV-3, and DENV-4). Infection with one serotype confers lifelong homotypic immunity, but only short term (approximately three to six months) cross protection against heterotypic serotypes.

[0004] The risk of severe disease is greatest during secondary, heterotypic infections in subjects with more than one circulating serotype. There is evidence that prior infection with one type can produce an antibody response that can intensify, or enhance, the course of disease during a subsequent infection with a different serotype. The possibility that vaccine components could elicit enhancing antibody responses, as opposed to protective responses, has been a major concern in designing and testing vaccines to protect against dengue infections. There is thus a

need for a vaccine that may be effective against different serotypes and which does not enhance the course of the DENV infection.

SUMMARY OF THE INVENTION

[0005] Described here is a method of forming a chimeric protein, comprising the steps of providing a yellow fever virus 17-D envelope protein having SEQ ID No. 1; providing a dengue fever virus envelope protein selected from the group consisting of SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, or SEQ ID No. 5; and substituting one or more of amino acids 1-11, 28-30, 32, 42, 44, 46, 70-81, 95-99, 110-115, 142-147, 149-157, 236-242, 304-324, 333, 335, 337, 350-352, 355, 356, 362-370, 377, 379, 386, 388-393 of SEQ ID No. 1 with the corresponding amino acid of the selected dengue fever virus envelope protein to create a chimeric envelope protein.

[0006] Also described here is a method of creating a treatment composition, comprising the steps of providing a portion of an envelope protein from a flavivirus; providing a dengue fever virus envelope protein selecting from the group consisting of SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, or SEQ ID No. 5; substituting a portion of the envelope protein amino acids of the flavivirus with a the corresponding envelope protein amino acids of the selected dengue fever virus to create a chimeric envelope protein; providing a pharmaceutically acceptable excipient; and mixing the chimeric envelope protein and the excipient.

[0007] Further described here is a chimeric protein, comprising: an envelope protein comprised of yellow fever virus 17-D envelope protein having SEQ ID No. 1, wherein selected amino acids of the yellow fever virus 17-D envelope protein are substituted with corresponding amino acids of dengue fever virus envelope protein selected from the group consisting of SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, or SEQ ID No. 5.

[0008] Also described here is a composition for treatment of dengue fever virus, comprising: a chimeric envelope protein comprised of a flavivirus envelope protein, wherein selected amino acids of the flavivirus envelope protein are substituted with corresponding amino acids of dengue fever virus envelope protein selected from the group consisting of SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, or SEQ ID No. 5; and a pharmaceutically acceptable excipient.

BRIEF DESCRIPTION OF THE DRAWINGS

[0009] FIG. 1 shows the results of ELISA assays with immobilized virus envelope glycoproteins, patient sera, and monoclonal antibodies from DENV infected patients.

[0010] FIG. 2 shows the results of immunofluorescence assays with DENV-1 to 4 infected LLC-MK-2 cells exposed to monoclonal antibodies from DENV infected patients.

[0011] FIG. 3 shows the results of neutralization assays with DENV-1 to 4 infected LLC-MK-2 cells, patient sera, monoclonal antibodies from DENV infected patients.

[0012] FIG. 4 shows the viral uptake results from enhancement assays performed in the presence of monoclonal antibodies from DENV infected patients.

[0013] FIG. 5 shows the results from virus-cell binding inhibition assays performed in the presence of monoclonal antibodies from DENV infected patients.

[0014] FIG. 6 shows the results from virus-liposome binding inhibition assays performed in the presence of monoclonal antibodies from DENV infected patients.

[0015] FIG. 7 shows the results from Western blot assays performed with purified DENV-2 and probed with monoclonal antibodies from DENV infected patients.

[0016] FIG. 8 shows the results from Western blot assays performed with soluble protein E and probed with monoclonal antibodies from DENV infected patients.

[0017] FIG. 9 shows the results from epitope mapping and gives the dissociation constants of monoclonal antibodies from DENV infected patients to soluble protein E.

[0018] FIG. 10 shows the results from binding competition ELISA assays with monoclonal antibodies from DENV infected patients and 4G2.

[0019] FIG. 11 shows the results from Western blot assays performed with DI/II and DIII and probed with monoclonal antibodies from DENV infected patients.

[0020] FIG. 12 shows the residues for monoclonal antibodies from DENV infected patients mapped to E protein crystal structure.

[0021] FIG. 13 shows an amino acid sequence alignment of DENV-1 to 4 and the yellow fever 17-D envelope protein.

[0022] FIG. 14 shows the protein crystal structure of the DENV envelope protein and demonstrates the location of the fusion loop (black) and 5Å (green) and 14Å (teal) surrounding amino acids.

DETAILED DESCRIPTION OF THE INVENTION

[0023] An increasing problem for public health officials has been the occurrence of severe complications arising from dengue viral infection. Both dengue hemorrhagic fever (DHF) and shock syndromes (DSS) are clinical outcomes related to the presence of pre-existing immunity to a heterologous dengue virus serotype. Dengue Haemorrhagic Fever is initially characterized by a minor febrile illness lasting 3-5 days. The patient may deteriorate at defervescence into the next phase of the syndrome with hemostatic disorders, and increased vascular permeability frequently accompanied by internal bleeding and shock. As many as 1.5 million children are reported to have been hospitalized with 33,000 deaths from this syndrome since it was first recognized in Thailand (World Health Organization 1989). DHF/DSS has since continued to persist, and outbreaks can pose major problems to public health in many countries.

[0024] Dengue virus (DENV) is a mosquito-transmitted virus and is expanding in geographic range and also in disease severity. There are four distinct serotypes of dengue that cause similar disease symptoms, serotypes 1-4. Infection with a single serotype results in an immune response that is protective against that same serotype, but causes a cross-reactive antibody response against the other serotypes (and other flaviviruses as well). Epidemiological studies have shown that the presence of cross-reactive antibodies correlates with a more severe disease outcome during subsequent infections with a different serotype. The mechanism for this effect appears to be an antibody-dependent enhancement of infection of macrophage and macrophage like cells that express Fc receptors. These cells are normally not infected efficiently by dengue, but become highly infectable in the presence of dengue virus binding antibodies that then target the virus particles

directly to the macrophages through the interaction of the antibody heavy chains and the cellular Fc receptors.

[0025] Studies have attempted to determine the human antibody response against dengue virus by characterizing human anti-dengue monoclonal antibodies. Prior to this work, most immunological studies on dengue infections had been conducted in mice, which are not a natural host for dengue and which produce a very different antibody response. One of the conclusions to come out of the human studies is that the dominant human antibody response against the dengue virus surface proteins, membrane (prM and M) and envelope (E, soluble envelope protein, sE), is non-neutralizing and cross reactive against the four serotypes of dengue. These non-neutralizing, cross-reactive antibodies are the primary cause of the antibody dependent enhancement of disease. This presents problems for the development of a dengue vaccine that uses the entire prM and E proteins. Even if a vaccine formulation using full length prM and E can induce a broad neutralizing response against all four serotypes, when the neutralizing antibody response wanes over time, the dominant non-neutralizing response will remain and prime vaccine recipients for severe disease if they are ever infected again. It is not yet clear how long the neutralizing vaccine response would endure or when vaccine recipients might become at risk for disease enhancement, but there are few examples of vaccines that induce lifelong protection.

[0026] The invention relates to a chimeric protein and methods for producing a chimeric protein for immunizing an individual against dengue and dengue clinical outcomes, and for treating an individual susceptible to infection or infected with dengue virus. In some embodiments, the chimeric protein could be used to create a treatment composition for an infected individual, while in others the chimeric protein could be used to produce a live attenuated vaccine, or a subunit vaccine that is not replicative.

[0027] The chimeric protein is created by substituting a portion of yellow fever virus (YFV) envelope protein, *Flavivirus yellow fever virus,* with a portion of any of the strains of dengue virus (DENV) envelope protein, *Flavivirus dengue virus*. In one embodiment, the chimeric protein of the invention is created using YFV 17D strain envelope protein. Although the example is limited to YFV envelope protein, in other embodiments it is envisioned the chimeric protein may be created using the envelope protein of any flavivirus, for example West Nile

Virus, St. Louis encephalitis, Dengue Fever virus, Japanese encephalitis, and Kunjin virus, and substituting any of the four strains of DENV envelope protein.

[0028] YFV 17D strain envelope protein has the following sequence, identified as SEQ ID No. 1:

AHCIGITDRDFIEGVHGGTWVSATLEQDKCVTVMAPDKPSLDISLETVAIDRPAEVRKVC
YNAVLTHVKINDKCPSTGEAHLAEENEGDNACKRTYSDRGWGNGCGLFGKGSIVACA
KFTCAKSMSLFEVDQTKIQYVIRAQLHVGAKQENWNTDIKTLKFDALSGSQEVEFIGYG
KATLECQVQTAVDFGNSYIAEMETESWIVDRQWAQDLTLPWQSGSGGVWREMHHLVE
FEPPHAATIRVLALGNQEGSLKTALTGAMRVTKDTNDNNLYKLHGGHVSCRVKLSALT
LKGTSYKICTDKMFFVKNPTDTGHGTVVMQVKVSKGAPCRIPVIVADDLTAAINKGILV
TVNPIASTNDDEVLIEVNPPFGDSYIIVGRGDSRLTYQWHKEGSSIGKLFTQTMKGVERL
AVMGDTAWDFSSAGGFFTSVGKGIHTVFGSAFQGLFGGLNWITKVIMGAVLIWVGINT
RNMTMSMSMILVGVIMMFLSLGVGA

[0029] DENV strain 1 envelope protein has the following sequence, identified as SEQ ID No. 2:

MRCVGIGSRDFVEGLSGATWVDVVLEHGSCVTTMAKDKPTLDIELLKTEVTNPAVLRK
LCIEAKISNTTTDSRCPTQGEATLVEEQDANFVCRRTFVDRGWGNGCGLFGKGSLITCA
KFKCVTKLEGKIVQYENLKYSVIVTVHTGDQHQVGNESTEHGTTATITPQAPTXEIQLTD
YGALTLDCSPRTGLDFNEMVLLTMKEKSWLVHKQWFLDLPLPWTSGASTSQETWNRQ
DLLVTFKTAHAKKQEVVVLGSQEGAMHTALTGATEIQTSGTTTIFAGHLKCRLKMDKL
TLKGMSYVMCTGSFKLEKEVAETQHGTVLVQIKYEGTDAPCKIPFSTQDEKGVTQNGR
LITANPIVTDKEKPVNIEAEPPFGESYIVIGAGEKALKLSWFKKGSSIGKMFEATARGARR
MAILGDTAWDFGSIGGVFTSVGKLVHQIFGTAYGVLFSGVSWTMKIGIGVLLTWLGLNS
RSTSLSMTCIAVGLV TLYLGVMVQA

[0030] DENV strain 2 envelope protein has the following sequence, identified as SEQ ID No. 3:

MRCIGISNRDFVEGVSGGSWVDIVLEHGSCVTTMAKNKPTLDFELIKTEAKQPATLRKY
CIEAKLTNTTTESRCPTQGEPSLNEEQDKRFVCKHSMVDRGWGNGCGLFGKGGIVTCA
MFTCKKNMEGKXVQPENLEYTIVITPHSGEEHAVGNDTGKHGKEIKITPQSSITEAELTG
YGTVTMECSPRTGLDFNEMVLLQMEXKAWLVHRQWFLDLPLPWLPGADTQGSNWIQK

ETLVTFKNPHAKKQDVVVLGSQEGAMHTALTGATEIQMSSGNLLFTGHLKCRLRMDKL
QLKGMSYSMCTGKFKXVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIMDLEKRHVLGRLI
TVNPIVTEKDSPVNIEAEPPFGDSYIIIGVEPGQLKLNWFKKGSSIGQMFETTMRGAKRM
AILGDTAWDFGSLGGVFTSIGKALHQVFGAIYGAAFSGVSWTMKILIGVIITWIGMNSRS
TSLSVSLVLVGVVTLYLGVMVQA

[0031] DENV strain 3 envelope protein has the following sequence, identified as SEQ ID No. 4:

MRCVGVGNRDFVEGLSGATWVDVVLEHGGCVTTMAKNKPTLDIELQKTEATQLATLR
KLCIEGKITNITTDSRCPTQGEAXLPEEQDQNYVCKHTYVDRGWGNGCGLFGKGSLVTC
AKFQCLEPIEGKVVQYENLKYTVIITVHTGDQHQVGNETQGVTAEITPQASTTEAILPEY
GTLGLECSPRTGLDFNEMILLTMKNKAWMVHRQWFFDLPLPWTSGATTETPTWNRKEL
LVTFKNAHAKKQEVVVLGSQEGAMHTALTGATEIQNSGGTSIFAGHLKCRLKMDKLEL
KGMSYAMCTNTFVLKKEVSETQHGTILIKVEYKGEDXPCKIPFSTEDGQGKAHNGRLIT
ANPVVTKKEEPVNIEAEPPFGESNIVIGIGDNALKINWYKKGSSIGKMFEATARGARRMA
ILGDTAWDFGSVGGVLNSLGKMVHQIFGSAYTALFSGVSWVMKIGIGVLLTWIGLNSK
NTSMSFSCIAIGIITLYLGAVVQA

[0032] DENV strain 4 envelope protein has the following sequence, identified as SEQ ID No. 5:

MRCVGVGNRDFVEGVSGGAWVDLVLEHGGCVTTMAQGKPTLDFELTKTTAKEVALLR
TYCIEASISNITTATRCPTQGEPYLKEEQDQQYICRRDVVDRGWGNGCGLFGKGGVVTC
AKFSCSGKITGNLVQIENLEYTVVVTVHNGDTHAVGNDTSNHGVTATITPRSPSVEVKLP
DYGELTLDCEPRSGIDFNEMILMKMKKKTWLVHKQWFLDLPLPWTAGADTSEVHWNY
KERMVTFKVPHAKRQDVTVLGSQEGAMHSALAGATEVDSGDGNHMFAGHLKCKVRM
EKLRIKGMSYTMCSGKFSIDKEMAETQHGTTVVKVKYEGAGAPCKVPIEIRDVNKEKV
VGRVISSTPLAENTNSVTNIELEPPFGDSYIVIGVGNSALTLHWFRKGSSIGKMFESTYRG
AKRMAILGETAWDFGSVGGLFTSLGKAVHQVFGSVYTTMFGGVSWMIRILIGFLVLWIG
TNSRNTSMAMTCIAVGGITLFLGFTVQA

[0033] FIG 13 shows an alignment of the YFV 17D strain envelope protein of and all four strains of DENV envelope protein. As used in this invention a "corresponding amino acid" is defined as follows. FIG 13 may be used to calculate which amino acids of the DENV envelope

protein corresponds to the amino acid of the YFV envelope protein. For example, FIG 13 shows that the first amino acid of YFV envelope protein, alanine, corresponds to the first amino acid of all four strains of DENV envelope protein, methionine. By way of a further example, FIG 13 may also be used to calculate that the 160th amino acid of the YFV envelope protein, lysine, corresponds to the following amino acids of the four strains of DENV envelope protein:

DENV strain	Amino acid position	Amino acid
DENV1	163rd	Threonine
DENV2	163rd	Lysine
DENV3	161st	Glutamic Acid
DENV4	163rd	Threonine

[0034] A similar amino acid alignment may be created by practitioners in the art with other flavivirus envelope proteins, for example with West Nile Virus, St. Louis encephalitis, Dengue Fever virus, Japanese encephalitis, and Kunjin virus envelope proteins. These amino acid alignments could be used to determine which amino acid of the flavivirus envelope protein corresponded to any of the four strains of DENV envelope protein.

[0035] Any or all of amino acids 1-11, 28-30, 32, 42, 44, 46, 70-81, 95-99, 110-115, 142-147, 149-157, 236-242, 304-324, 333, 335, 337, 350-352, 355, 356, 362-370, 377, 379, 386, 388-393 of YFV envelope protein, SEQ ID No. 1, may be substituted with the corresponding amino acid of the desired strain of DENV envelope (E) protein to create the chimeric protein of the invention. The substitution may be made according to methods known to practitioners in the art. For example, site directed mutagenesis of the envelope protein may be performed to create the chimeric protein. Briefly, this method makes use of a short mutant DNA primer that binds specifically to the region being changed, but contains one or a small number of specific base changes that will result in a coding change to substitute the new specifically desired amino acid. The bacterial plasmid with the E gene is replicated using PCR amplification to generate new full-

length mutant DNA strands. Then the original DNA strand is degraded, leaving only the remaining specifically mutated DNA strand.

[0036] The chimeric protein is created by substituting amino acids of YFV envelope protein proximal to the domain II fusion loop. As used in this invention, amino acids "proximal to" the domain II fusion loop are those amino acids which are near the domain II fusion loop of the YFV envelope protein, shown in FIG 13. In one embodiment, amino acids which are within 5Å of the fusion loop are proximal to the fusion loop. In another embodiment, those amino acids which are within 14Å of the fusion loop are proximal to the fusion loop are proximal to the fusion loop. Amino acids within 5Å and 14Å of the fusion loop are also shown in FIG 13.

[0037] The fusion loop is a structural feature of flavivirus envelope proteins that is found on the tip of domain II and is responsible for direct interaction of the envelope (E) protein with the target cell lipid membrane. During the infection process, the fusion loop is projected outward by a structural rearrangement of the E protein, resulting in the fusion loop "harpooning" into the target cell membrane. This interaction is critical for the subsequent membrane fusion step, mediated by a further E protein movement that pulls the cell and virus membranes together. As shown in FIG 13, the fusion loop is highly conserved in dengue and yellow fever viruses. The cysteine (C) at position 105 in the fusion loop forms a disulfide bond with the conserved cysteine (C) at position 74. This disulfide is important for the correct folding of the fusion loop. Amino acids within 5Å and 14Å of the fusion loop are important in YFV and DENV infection as well.

[0038] It is envisioned the chimeric protein may be used as a treatment composition for DENV infected individuals. It is further envisioned the chimeric protein may be used to create a treatment composition for preventing infection, or a vaccine effective against one or all four strains of DENV.

[0039] In an embodiment, the chimeric protein, such as for use in a vaccine, may use a small portion of the yellow fever virus (YFV) 17D vaccine strain envelope protein to be replaced by the corresponding portion from the dengue virus envelope protein. For an attenuated vaccine, the vaccine may use a replication competent YFV with the DENV/YFV hybrid E protein. An attenuated vaccine is created by reducing the virulence of a pathogen like YFV, but still keeping it viable (or "live"). Attenuation takes an infectious agent and alters it so that it

becomes harmless or less virulent. These vaccines contrast to those produced by "killing" the virus (inactivated vaccine). Alternately, the invention could be used to develop a subunit vaccine that is not replicative. Rather than introducing an inactivated or attenuated micro-organism to an immune system (which would constitute a "whole-agent" vaccine), a fragment of it can create an immune response, and relate to producing a subunit vaccine.

[0040] There is also presented by the invention methods for controlling a flavivirus entry into a cell, and methods of treating and preventing flaviviruses infections, together with vaccine and pharmaceutical compositions. Along with dengue, the family Flaviviridae contains at least 70 arthropod-transmitted viruses, many of which infect humans and other vertebrates. Subgroups of the Flaviviridae family include West Nile, Japanese Encephalitis, tick borne encephalitis, etc. the Japanese encephalitis serocomplex, includes West Nile Virus, St. Louis encephalitis, Murray Valley encephalitis, kunjin and other viruses. As an alternate approach to the use of a small portion of the yellow fever virus (YFV) 17D vaccine strain envelope protein to be replaced by the corresponding portion from the dengue virus envelope protein, the invention may swap out the dengue neutralizing epitopes into any other related flavivirus.

[0041] All flaviviruses, including West Nile Virus, St Louis encephalitis, dengue, Japanese encephalitis, yellow fever and kunjin viruses share similar size, symmetry and appearance. Despite the fact that flaviviruses may use different process to enter a host cell, such as endocytotis (described for West Nile Virus and Kunjin Virus) and direct fusion of the cell (described for dengue and Encephalitis Virus), entry of all flaviviruses into the host-cell involves an interaction between the virus and a receptor of the cell.

[0042] As the viral envelope protein of flaviviruses plays a role in mediating virus-host cellular receptor interaction, the invention contemplates use of dengue neutralizing epitopes into any other related flavivirus.

[0043] The invention creates a vaccine that will induce broadly protective antibodies against dengue virus and reduce the induction of non-neutralizing antibodies that will cause enhancement. The invention relates to a vaccine and methods of producing a vaccine using information from defining the regions of the E protein that are responsible for inducing a neutralizing antibody response to dengue. Neutralizing antibodies can produce the infection enhancing effect, but they only do so at sub-

neutralizing concentrations, while non-neutralizing antibodies produce enhancement at all concentrations. The invention recognizes that if the human antibody response could be shifted away from a non-neutralizing response and towards a neutralizing response, this could considerably reduce the risk of post-vaccination disease enhancement. The invention provides a vaccine that substantially reduces the risk of inducing an enhancing antibody response. A vaccine formulation could accomplish this by only including the dengue E protein epitopes that induce a neutralizing response, and not including the epitopes that produce a non-neutralizing response. The invention describes a common class of human, broadly neutralizing monoclonal antibodies and identifies their binding epitope, allowing the design a vaccine formulation. The invention has confirmed the envelope portion from dengue virus that will be replaced in the yellow fever vaccine strain, and contemplates using common techniques to allow the resulting chimeric viruses to grow well enough to provide a suitable vaccine response.

[0044] The broadly neutralizing monoclonal antibodies were determined from each of three dengue patients infected in Jamaica, Singapore, and Myanmar, at time points two weeks, two months, and two years post infection. These antibodies (4.8A, D11Ckl, and 1.6D) show neutralization activity against all four serotypes of dengue and recognize a common epitope consisting of the E protein fusion loop and nearby regions. They target this region because they interfere with the binding of a previously characterized mouse monoclonal antibody that is known to target the fusion loop (4G2) and they interfere with each other's binding.

[0045] Confirmation of the fusion loop as a target comes from mechanistic experiments. These antibodies do not interfere with virus: cell binding, but do inhibit the ability of virus to fuse with liposomes. There is further defined their epitopes through binding experiments with a large panel of E protein mutants. Mutations in the E protein that prevent binding of these antibodies map to locations in and near to the fusion loop.

[0046] These antibodies (4.8A, D11Ckl, and 1.6D) do not show strong neutralization activity against yellow fever virus, indicating that the yellow fever virus E protein lacks the important amino acid sequences that are recognized by these antibodies. Because the fusion loops of dengue and yellow fever are identical, the important amino acid positions lie outside of the fusion loop.

An amino acid sequence alignment of the dengue and yellow fever E proteins shows the differences between dengue and yellow fever that are responsible for antibody recognition.

[0047] Exchange of these dengue specific amino acid sequences into the yellow fever E protein will yield a chimeric E protein that will induce and be recognized by neutralizing antibodies against dengue. This chimeric E protein can be used as a modification to the yellow fever 17-D vaccine, one of the most successful vaccines ever developed.

[0048] Fig. 1 establishes DENV specificity and broad reactivity of patient sera.

[0049] Fig. 2 establishes specificity for DENV antigens in the context of a cell.

[0050] Fig. 3 establishes neutralization activity of patient sera and of monoclonal antibodies from DENV infected patients.

[0051] Fig. 4 establishes that enhancing concentrations correlate with binding affinity to DENY-1 to 4, and also shows neutralization.

[0052] Fig. 5 rules out binding inhibition as mechanism.

[0053] Fig. 6 establishes mechanism as fusion inhibition for monoclonal antibody D11Ckl.

[0054] Fig. 7 shows that the monoclonal antibodies from DENV infected patients bind to protein consistent with size of E and the epitope is conformationally sensitive.

[0055] Fig. 8 confirms that the monoclonal antibodies from DENV infected patients bind to E, specifically to the ectodomain.

[0056] Fig. 9 determines how tightly the monoclonal antibodies from DENV infected patients bind to E protein.

[0057] Fig. 10 establishes that monoclonal antibodies from DENV infected patients compete for same domain as 4G2, a known fusion loop binder.

[0058] Fig. 11 establishes that monoclonal antibodies from DENV infected patients bind to DI/II consistent with fusion inhibition and competition assays.

[0059] Fig. 12 confirms monoclonal antibody 4.8A epitope as fusion loop and/or vicinity.

[0060] Fig. 13 highlights the amino acids in yellow fever virus 17-D envelope protein which may be substituted with the DENV-1 to 4 envelope amino acids to create a chimeric E protein. It highlights the desirable amino acid substitution locations, as well as proposed amino acid substitutions.

[0061] Fig. 14 shows the protein crystal structure of the DENV envelope protein and demonstrates the location of the fusion loop at 10 and 5Å at 12 and 14Å at 14 surrounding amino acids.

[0062] In still another aspect, the invention includes a vaccine for immunizing an individual against dengue hemorrhagic fever and/or dengue shock syndrome. The vaccine includes one or more peptides of the type described, in a pharmaceutically acceptable adjuvant.

[0063] It should be recognized that the invention provides a dengue vaccine and methods using a small portion of the yellow fever virus 17D vaccine strain envelope protein (or other related faviviruses) to be replaced by the corresponding portion from the dengue virus envelope protein. This creates a chimeric protein, such as for use in a vaccine, that will induce broadly protective antibodies against dengue virus and reduce the induction of non-neutralizing antibodies that will cause enhancement. While the invention has been described with reference to certain embodiments, it will be understood by those skilled in the art that various changes may be made and equivalents may be substituted without departing from the scope of the invention. In addition, many modifications may be made to adapt a particular situation or material to the teachings of the invention without departing from its scope. Therefore, it is intended that the invention not be limited to any particular embodiment disclosed, but that the invention will include all embodiments falling within the scope of the appended claims.

What is claimed is:

1. A method of forming a chimeric protein, comprising the steps of:

providing a yellow fever virus 17-D envelope protein having SEQ ID No. 1;

providing a dengue fever virus envelope protein selected from the group consisting of SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, or SEQ ID No. 5; and

substituting one or more of amino acids 1-11, 28-30, 32, 42, 44, 46, 70-81, 95-99, 110-115, 142-147, 149-157, 236-242, 304-324, 333, 335, 337, 350-352, 355, 356, 362-370, 377, 379, 386, 388-393 of SEQ ID No. 1 with the corresponding amino acid of the selected dengue fever virus envelope protein to create a chimeric envelope protein.

2. The method of claim 1, wherein the substituted amino acids comprise the amino acids proximal to a Domain II fusion loop.

3. The method of claim 2, wherein the substituted amino acids comprise the amino acids within 5Å of the fusion loop.

4. The method of claim 2, wherein the substituted amino acids comprise the amino acids within 14Å of the fusion loop.

5. A method of treating a dengue fever virus infection, the method comprising the step of administering the chimeric protein formed as in claim 1.

6. A method of creating a treatment composition, comprising the steps of:

providing a portion of an envelope protein from a flavivirus;

providing a dengue fever virus envelope protein selecting from the group consisting of SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, or SEQ ID No. 5;

substituting a portion of the envelope protein amino acids of the flavivirus with a the corresponding envelope protein amino acids of the selected dengue fever virus to create a chimeric envelope protein;

providing a pharmaceutically acceptable excipient; and

mixing the chimeric envelope protein and the excipient.

7. The method of claim 6, where the flavivirus is selected from the group consisting of West Nile Virus, St. Louis encephalitis, Dengue Fever virus, Japanese encephalitis, Yellow Fever virus, and Kunjin virus.

8. The method of claim 6, wherein the substituted amino acids comprise the amino acids proximal to a fusion loop.

9. The method of claim 6, wherein the substituted amino acids comprise the amino acids within 5Å of the fusion loop.

10. The method of claim 6, wherein the substituted amino acids comprise the amino acids within 14 Å of the fusion loop.

11. A method of treating a dengue fever virus infection, the method comprising the step of administering the chimeric protein composition formed as in claim 6.

11. A chimeric protein, comprising:

an envelope protein comprised of yellow fever virus 17-D envelope protein having SEQ ID No. 1, wherein selected amino acids of the yellow fever virus 17-D envelope protein are substituted with corresponding amino acids of dengue fever virus envelope protein selected from the group consisting of SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, or SEQ ID No. 5.

12. The chimeric protein of claim 11, wherein the substituted amino acids comprise the amino acids proximal to a Domain II fusion loop.

13. The chimeric protein of claim 11, wherein the substituted amino acids comprise the amino acids within 5Å of the fusion loop.

14. The chimeric protein of claim 11, wherein the substituted amino acids comprise the amino acids within 14Å of the fusion loop.

15. The chimeric protein of claim 11, wherein one or more of amino acids 1-11, 28-30, 32, 42, 44, 46, 70-81, 95-99, 110-115, 142-147, 149-157, 236-242, 304-324, 333, 335, 337, 350-352, 355, 356, 362-370, 377, 379, 386, and 388-393 of SEQ ID No. 1 are substituted with the corresponding amino acids of the selected dengue fever virus envelope protein.

16. A method of treating a dengue fever virus infection, the method comprising the step of administering the chimeric protein of claim 11.

17. A composition for treatment of dengue fever virus, comprising:

a chimeric envelope protein comprised of a flavivirus envelope protein, wherein selected amino acids of the flavivirus envelope protein are substituted with corresponding amino acids of

dengue fever virus envelope protein selected from the group consisting of SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, or SEQ ID No. 5; and

a pharmaceutically acceptable excipient.

18. The composition of claim 17, wherein the flavivirus is selected from the group consisting of West Nile Virus, St. Louis encephalitis, Dengue Fever virus, Japanese encephalitis, Yellow Fever virus, and Kunjin virus.

19. The composition of claim 17, wherein the substituted amino acids comprise the amino acids proximal to a fusion loop.

20. The composition of claim 17, wherein the substituted amino acids comprise the amino acids within 5Å of the fusion loop.

21. The composition of claim 17, wherein the substituted amino acids comprise the amino acids within 14Å of the fusion loop.

22. A method of treating a dengue fever virus infection, the method comprising the step of administering the chimeric protein of claim 11.

23. A method of treating a dengue fever virus infection, the method comprising the step of administering the composition of claim 17.

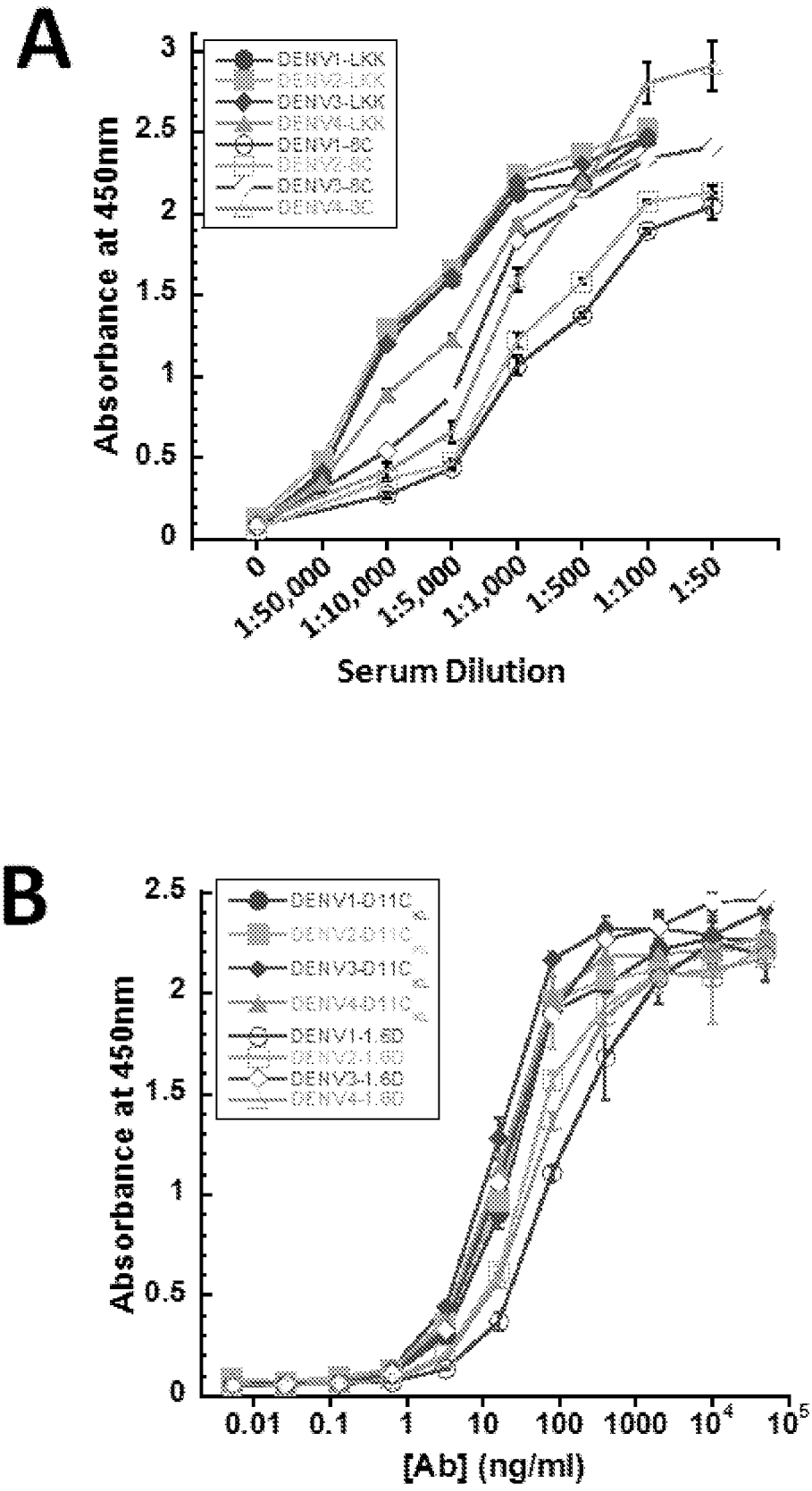
A
Absorbance at 450nm
Serum Dilution
0
1:50,000
1:10,000
1:5,000
1:1,000
1:500
1:100
1:50
B
Absorbance at 450nm
[Ab] (ng/ml)
0.01
0.1
1
10
100
1000

FIG. 1

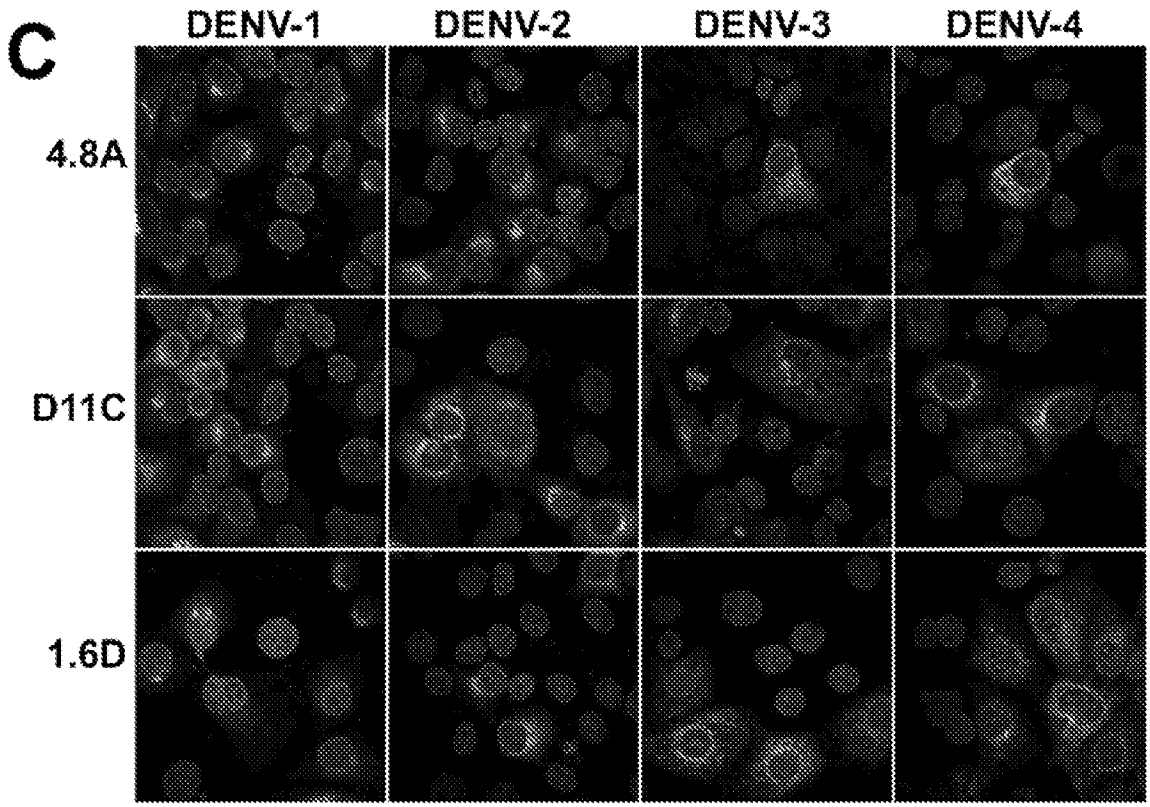

FIG. 2

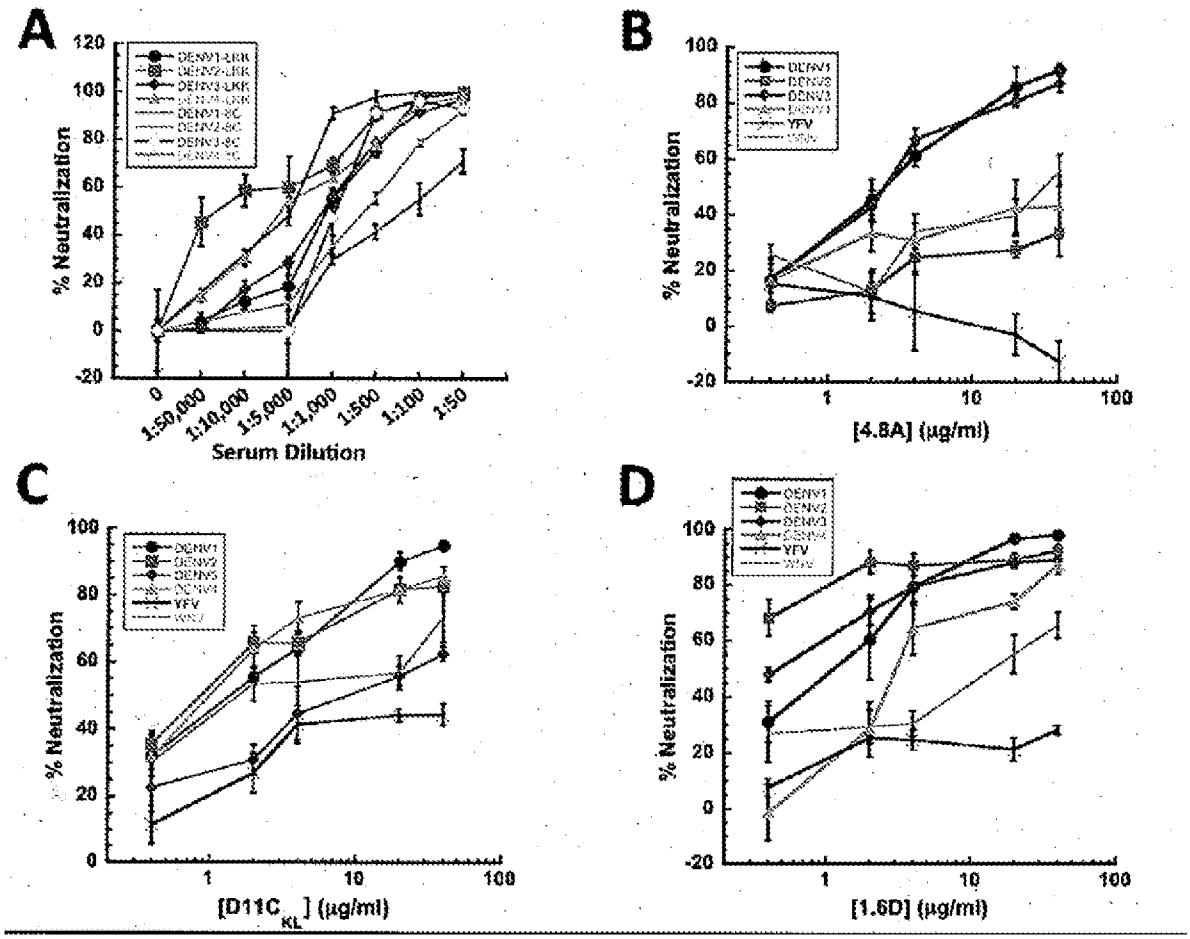
A
% Neutralization
Serum Dilution
0
1:50,000
1:10,000
1:5,000
1:1,000
1:500
1:100
1:50
B
% Neutralization
[4.8A] (µg/ml)
DENV1
DENV2
DENV3
YFV
C
% Neutralization
[D11C KL] (µg/ml)
DENV1
DENV2
DENV3
YFV
D
% Neutralization
[1.6D] (µg/ml)
DENV1
DENV2
DENV3
YFV

FIG. 3

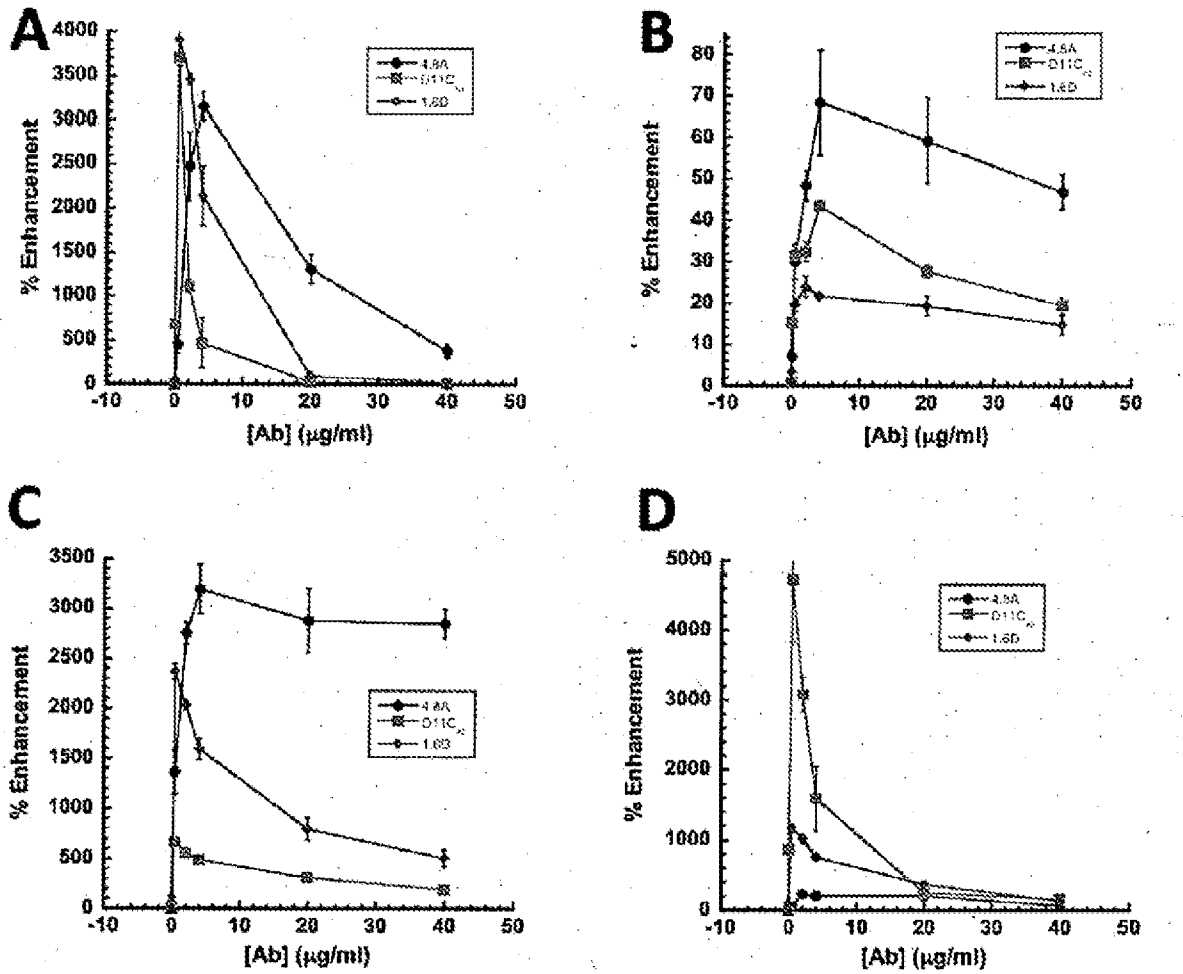

FIG. 4

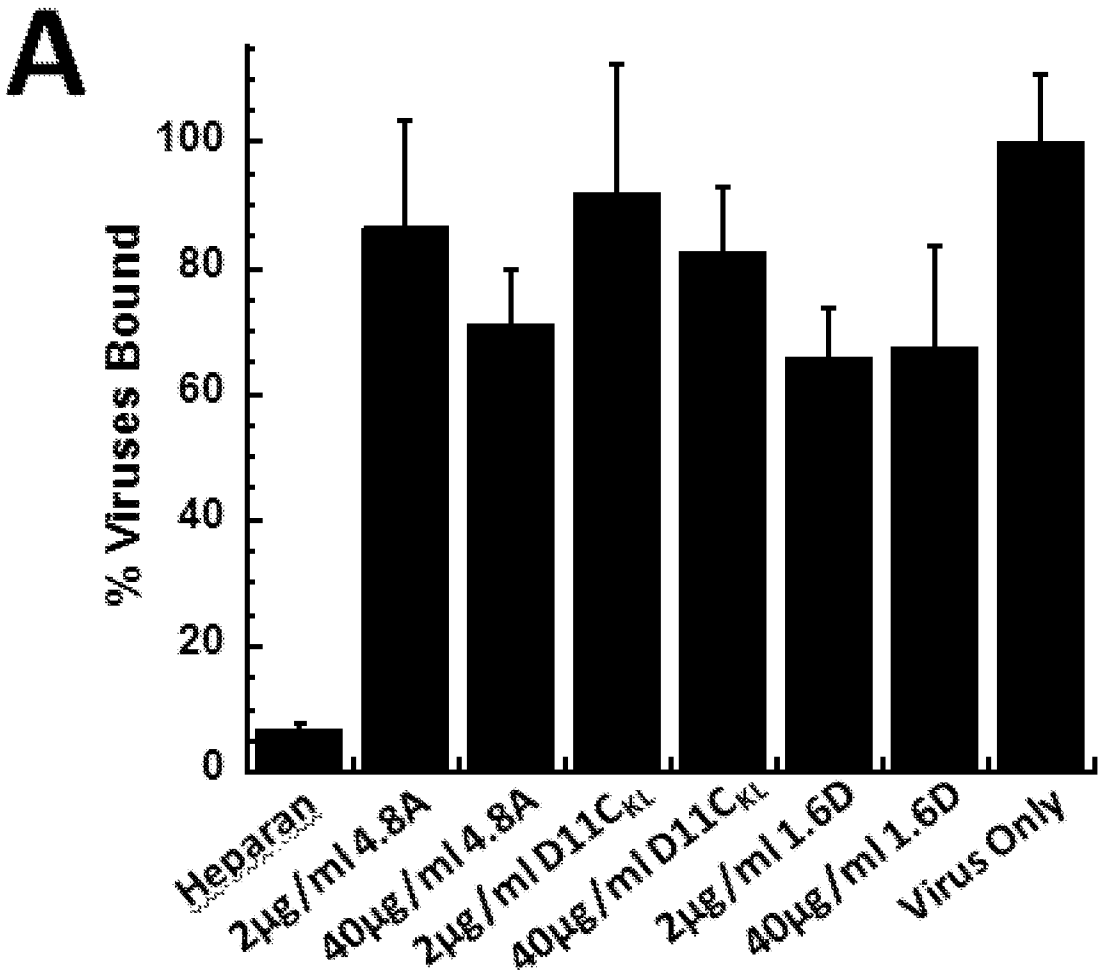
A
% Viruses Bound
0
20
40
60
80
100
Heparan
2μg/ml 4.8A
40μg/ml 4.8A
2μg/ml D11C$_{KL}$
40μg/ml D11C$_{KL}$
2μg/ml 1.6D
40μg/ml 1.6D
Virus Only

FIG. 5

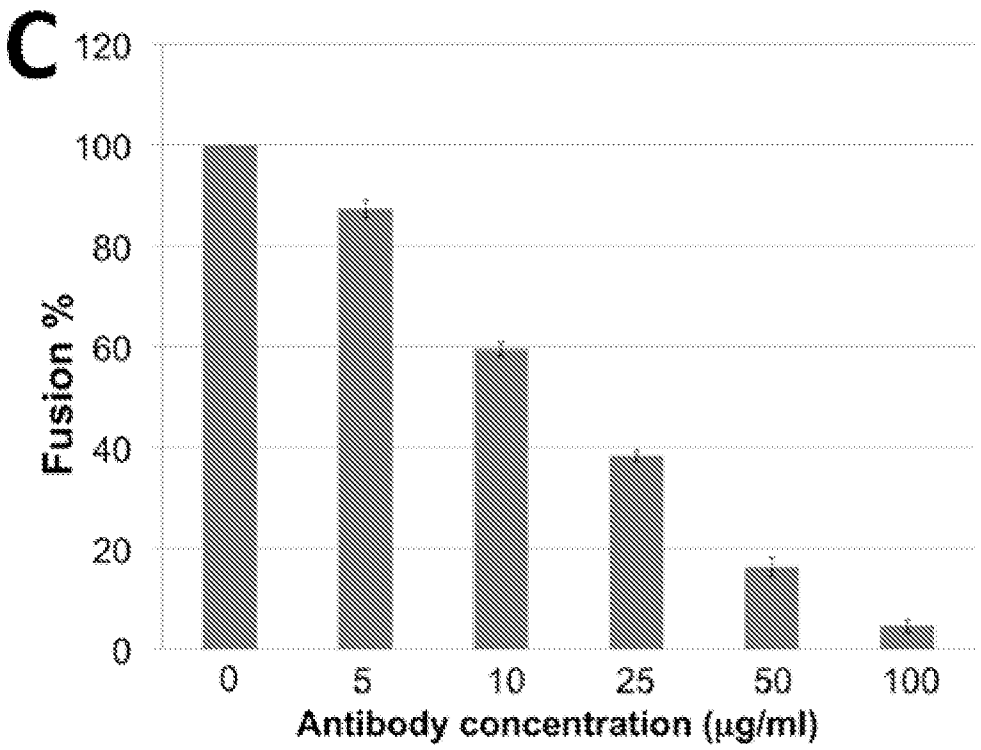
C
120
100
80
60
40
20
0
Fusion %
0
5
10
25
50
100
Antibody concentration (µg/ml)

FIG. 6

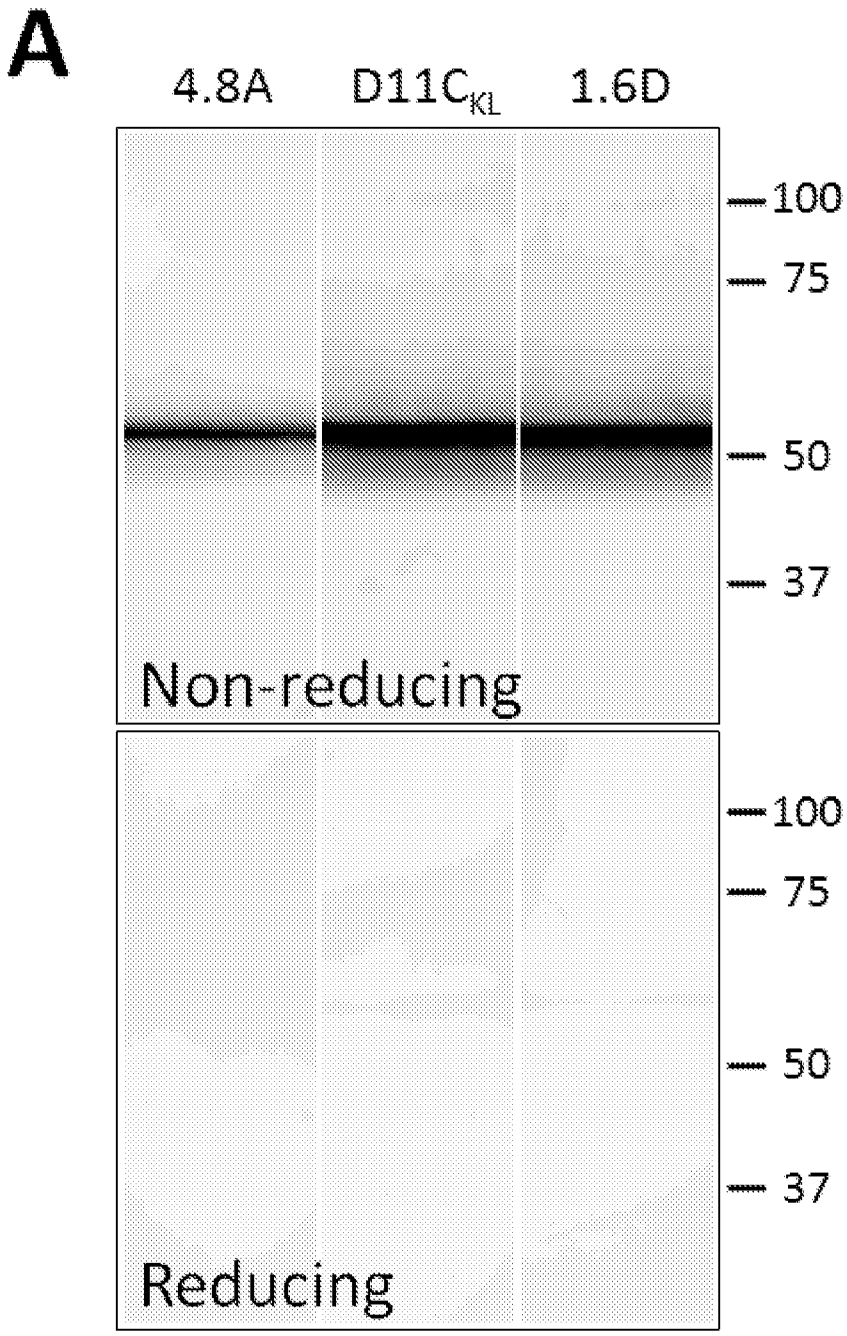
A
4.8A
D11C$_{KL}$
1.6D
100
75
50
37
Non-reducing
100
75
50
37
Reducing

FIG. 7

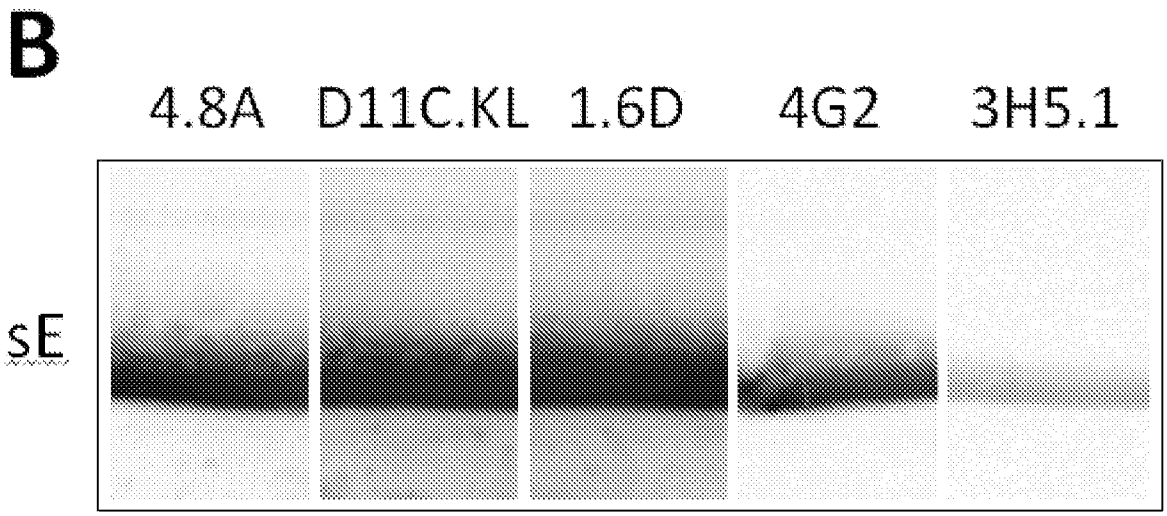

FIG. 8

	4.8A	D11C$_{KL}$	1.6D
DENV1	6.70175E-10	3.951E-10	2.75125E-10
DENV2	7.5382E-10	1.8205E-09	1.86987E-09
DENV3	1.43508E-09	3.293E-10	1.7884E-09
DENV4	1.15297E-09	4.7165E-10	1.619E-09

FIG. 9

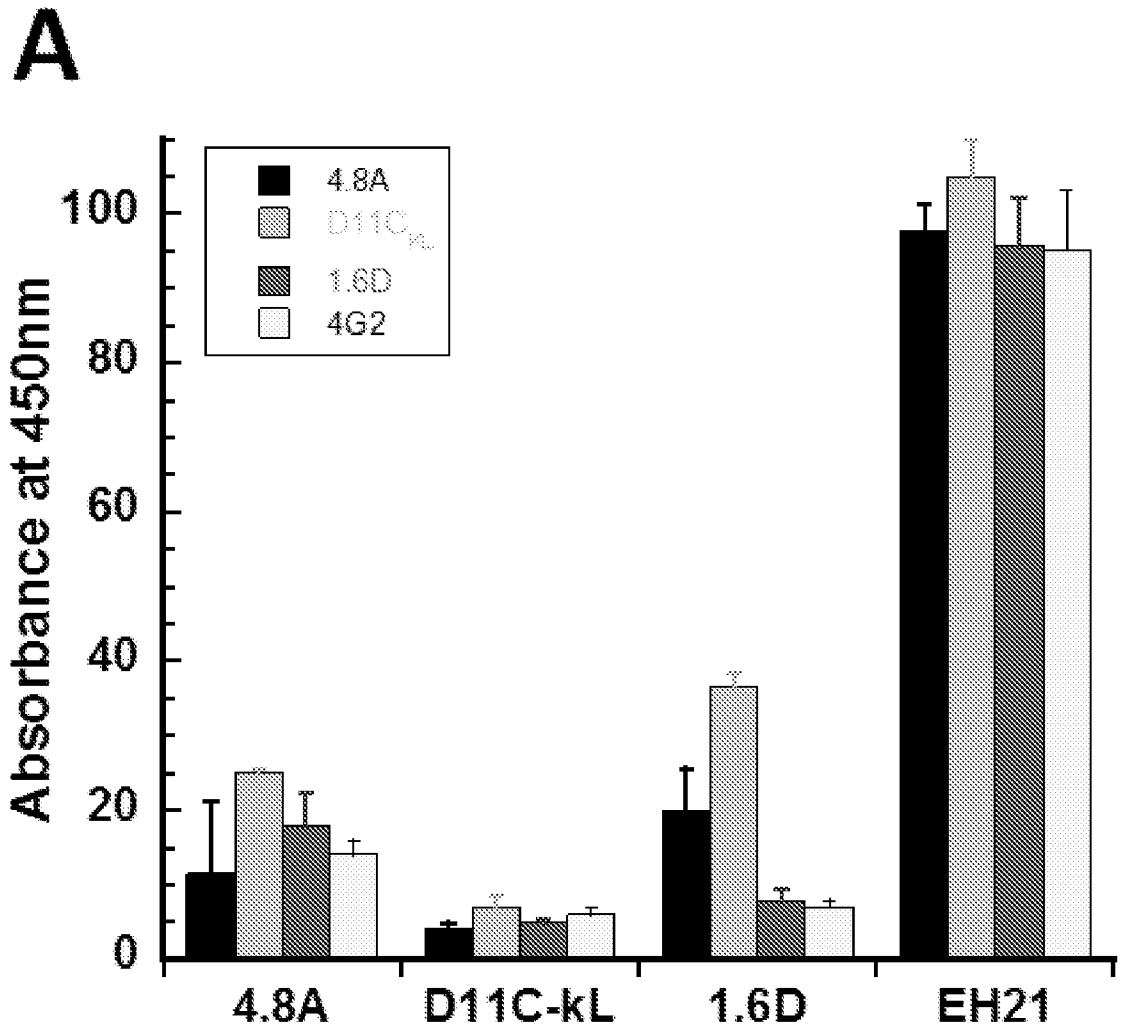
A
Absorbance at 450nm
0
20
40
60
80
100
4.8A
D11C-kL
1.6D
4G2
4.8A
D11C-kL
1.6D
EH21

FIG. 10

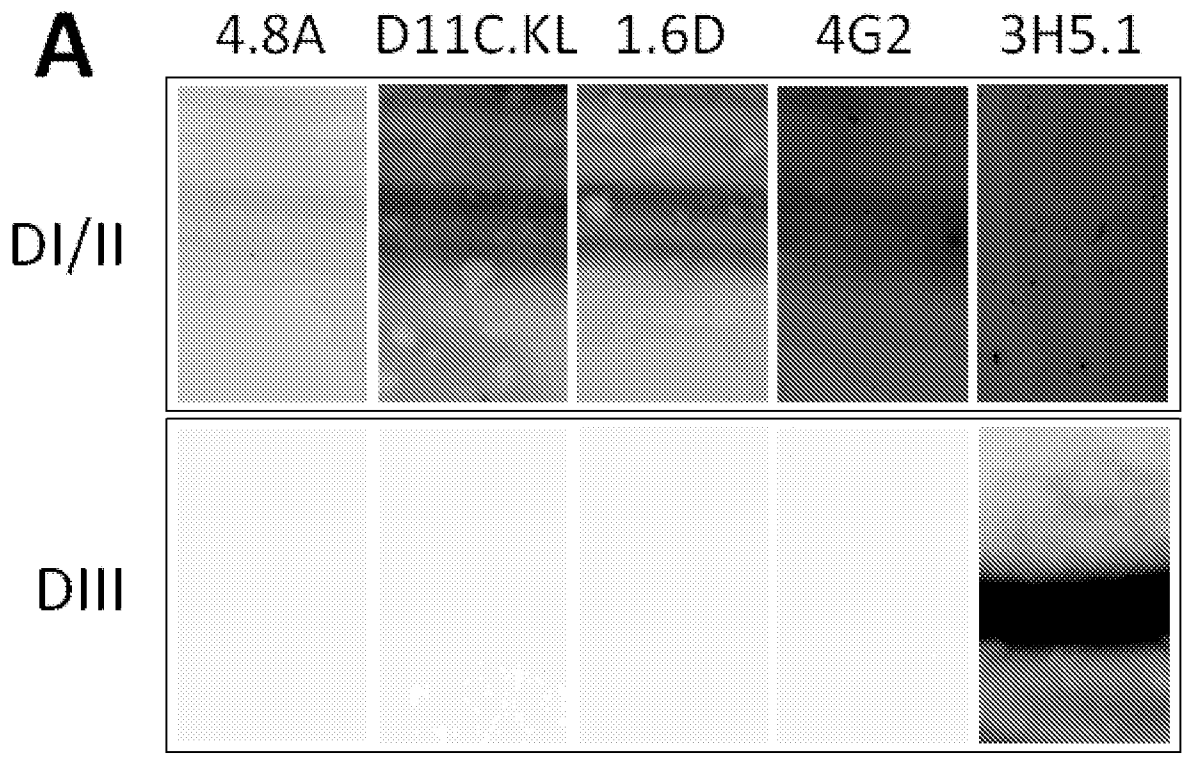

FIG. 11

B

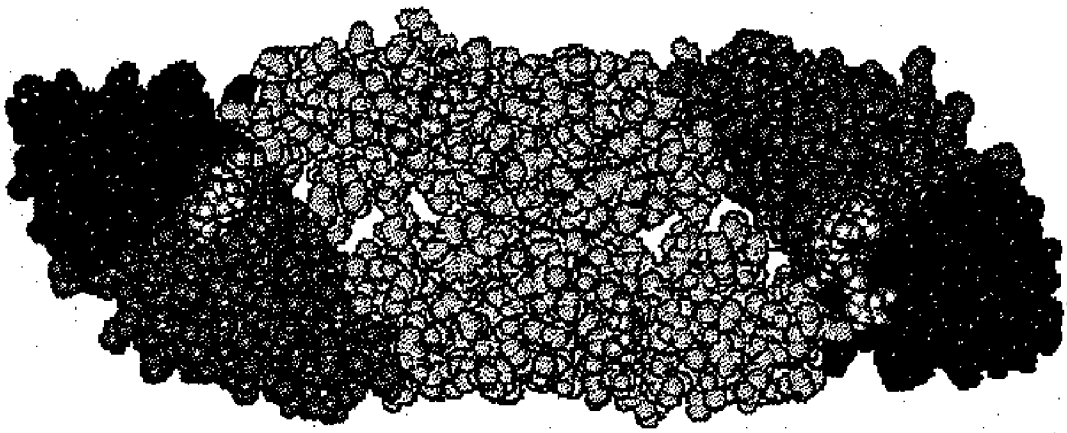

FIG. 12

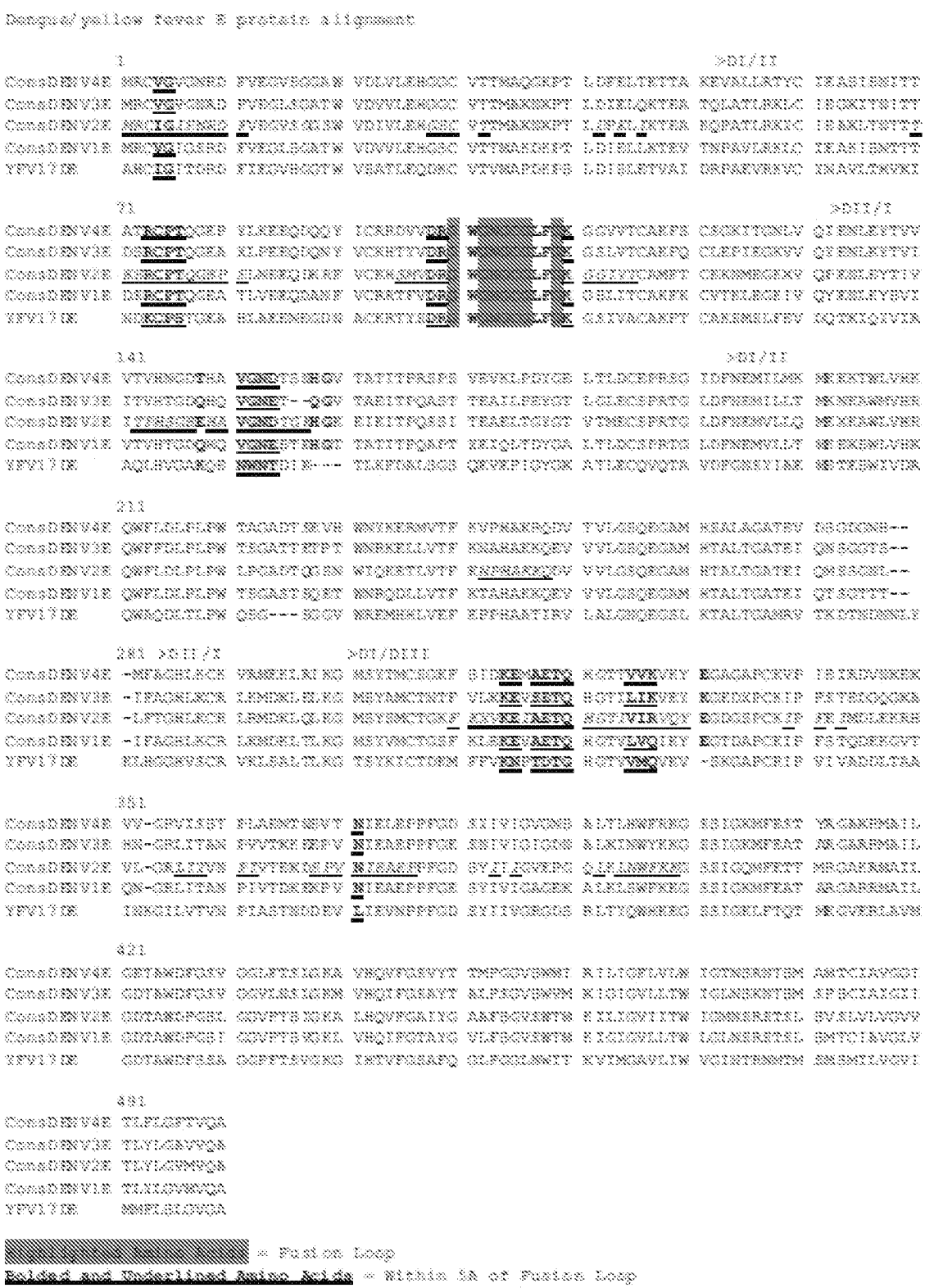
Dengue/yellow fever E protein alignment
= Fusion Loop
= Within 5A of Fusion Loop
= Within 14A of Fusion Loop (shown only for DENV2)
= Loss of binding position for 4.8A, D11Cb1, and 1.6D
4.8A W101, L107, Q148
D11Cb1 W101, G157, K325
1.6D W101, F108, Q156

FIG. 13

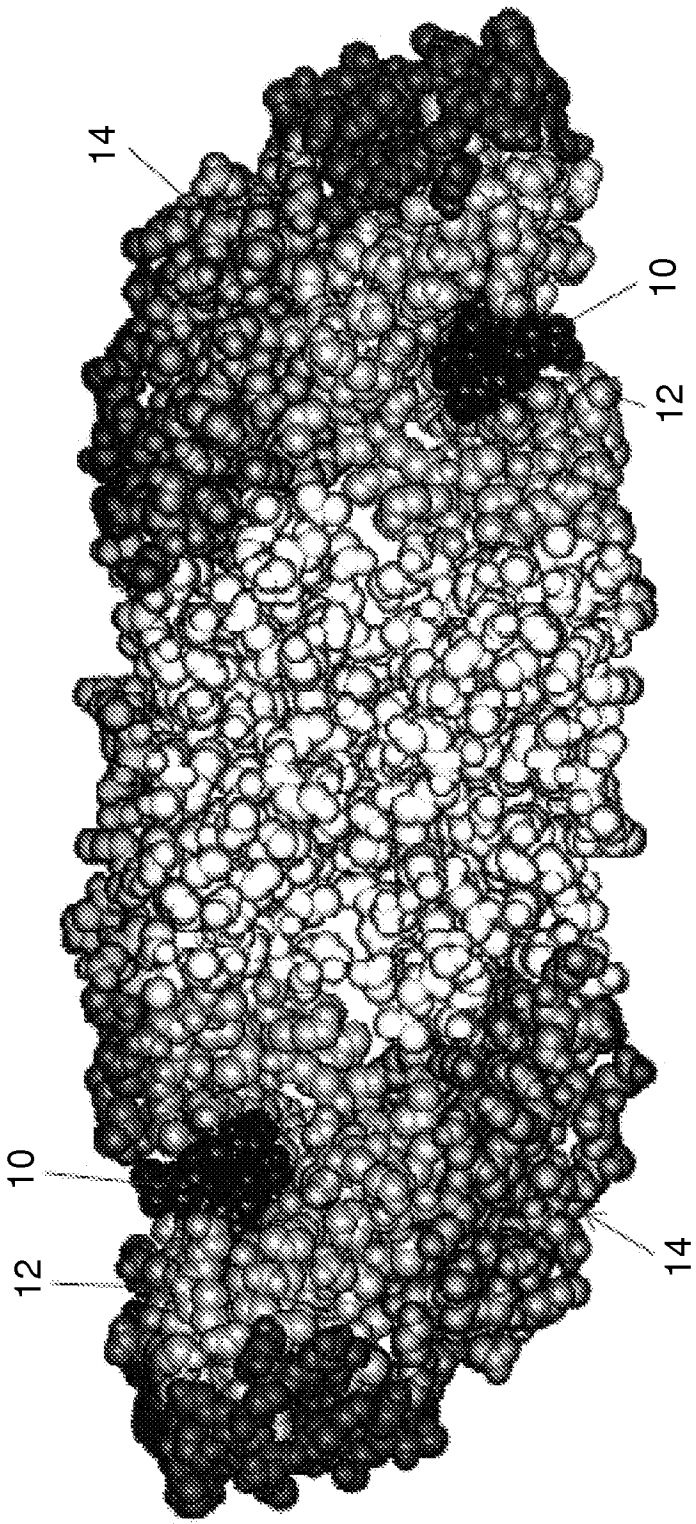

FIG. 14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2012/061893

A. CLASSIFICATION OF SUBJECT MATTER

C07K 19/00(2006.01)i, A61K 39/12(2006.01)i, C07K 14/18(2006.01)i, A61P 31/14(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K35/76; A61K38/00; A61K39/00; A61K39/002; A61K39/02; A61K39/12; A61K48/00; A61P31/04; A61P31/12; A61P33/00; A61P35/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

eKOMPASS(KIPO internal) & keywords: chimeric protein, yellow fever virus 17-D, dengue fever virus, envelope protein, substituting

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GUIRAKHOO, F. et al., 'Construction, Safety, and Immunogenicity in Nonhuman Primates of a Chimeric Yellow Fever-Dengue Virus Tetravalent Vaccine' Journal of Virology, August 2001, Vol. 75, No. 16, pp. 7290-7304, ISSN 0022-538X. See page 7291, left column; page 7292, left column; page 7293, left column; page 7294, right column; figure 1; table 1.	1-4,6-10,11b-15,17-21
A	US 2003-0194801 A1 (BONALDO, MIRNA C. et al.)16 October 2003 See claims 1, 4-5, 7 and 10.	1-4,6-10,11b-15,17-21
A	US 2003-0044773 A1 (KLEANTHOUS, HAROLD. et al.) 06 March 2003 See claims 13-15 and 17-25.	1-4,6-10,11b-15,17-21
A	VAN DER MOST, R. G. et al., 'Chimeric Yellow Fever/Dengue Virus as a Candidate Dengue Vaccine: Quantitation of the Dengue Virus-Specific CD8 T-Cell Response' Journal of Virology, September 2000, Vol. 74, No. 17, pp. 8094-8101, ISSN 0022-538X. See page 8095, left column, right column and page 8096, left column.	1-4,6-10,11b-15,17-21

☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 FEBRUARY 2013 (28.02.2013)	**28 FEBRUARY 2013 (28.02.2013)**
Name and mailing address of the ISA/KR Facsimile No. 82-42-472-7140	Authorized officer HEO, Joo Hyung Telephone No. 82-42-481-8150

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2012/061893

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of :

a. a sequence listing filed or furnished

- ☐ on paper
- ☒ in electronic form

b. time of filing or furnishing

- ☒ contained in the international application as filed
- ☐ filed together with the international application in electronic form
- ☐ furnished subsequently to this Authority for the purposes of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2012/061893

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 5, 11a, 16, 22,23
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 5, 11a, 16, 22, and 23 pertain to methods for treatment of the human body by therapy and thus relate to a subject matter which this International Searching Authority is not required, under Article 17(2)(a)(i) of the PCT and Rule 39.1(iv) of the Regulations under the PCT, to search.

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required addtional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2012/061893

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2005-0002968 A1 (MONATH, THOMAS P. et al.) 06 January 2005 See paragraphs [0091]-[0096]; claims 1-4, 6 and 8-10; table 5.	1-4,6-10,11b-15,17-21

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2012/061893

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2003-0194801 A1	16.10.2003	AP 200202685 D0	31.12.2002
		BR 0204470 A	08.09.2004
		CA 2408214 A1	19.09.2002
		EP 1366170 A1	03.12.2003
		GB 0105877 D0	25.04.2001
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		ZA 200209371 A	18.02.2004
US 2003-0044773 A1	06.03.2003	BR 0210907 A	21.12.2004
		CA 2448971 A1	27.12.2002
		CN 1551782 A	01.12.2004
		EP 1401859 A2	31.03.2004
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		JP 2005-501532 A	20.01.2005
		PL 371187 A1	13.06.2005
		US 7569383 B2	04.08.2009
		US 2011-0014229 A1	20.01.2011
		WO 02-102828 A2	27.12.2002
		WO 02-102828 A3	09.10.2003
US 2005-0002968 A1	06.01.2005	AU 2005-216248 A1	09.09.2005
		BR PI0508064 A	17.07.2007
		CA 2557136 A1	09.09.2005
		CN 1950499 A	18.04.2007
		EP 1755539 A2	28.02.2007
		EP 1755539 A4	21.01.2009
		IL 177667 D0	31.12.2006
		JP 2007-525226 A	06.09.2007
		KR 10-2006-0135844 A	29.12.2006
		NZ 549749 A	26.03.2010
		SG 150551 A1	30.03.2009
		WO 2005-082020 A2	09.09.2005
		WO 2005-082020 A3	22.12.2005