

**PRE-M/M EPITOPES OF DENGUE VIRUS, SYNTHETIC PEPTIDES,
CHIMERIC PROTEINS AND ITS USE.**

Technical branch

5 The present invention is in the field of biotechnology and relates to recombinant DNA techniques, in particular to the production of synthetic peptides coding for pre-M/M protein of Dengue virus serotype 2 and chimeric proteins which contain epitopes of pre-M/M protein of Dengue virus serotype
10 2 and 4.

The technical objective is to identify Pre-M/M neutralizing and protective epitopes, cross reactive for all dengue virus serotypes to obtain an immunogen for human vaccination.

Previous technique

15 Dengue virus belong to Flavivirus genus, family Flaviviridae (Westaway, E.G. et al. 1985. Flaviviridae. *Intervirology*. 24 p.183). It is an enveloped virus with a single RNA chain of positive polarity as genetic material, which codes for a polyprotein processed co- and post- transcriptionally by
20 cellular and viral proteases.

There are two structural proteins in the viral membrane: E (envelope) and M (membrane), while there are several copies of the other structural protein, C (capsid) forming the isometric nucleocapsid. Besides, at least seven non-
25 structural proteins have been identified (NS1, NS2a, NS2b, NS3, NS4a, NS4b, NS5).

Glycoproteins E and NS1 are individually able to offer active and passive protection against the homologous serotype of Dengue virus, while the highly conformational complexity of
30 the relevant epitopes is preserved. For this reason, recombinant eukaryotic cellular systems have been mainly selected for the immunological evaluation of these proteins, for example vaccinia virus (Bray, M. et al. 1989. Mice immunized with recombinant Vaccinia virus expressing dengue-4
35 structural proteins with or without nonstructural protein NS1

are protected against fatal dengue virus encephalitis. *J. Virol.* 63 p.2853) and baculovirus (Zhang, Y. M. et al. 1988. Immunization of mice with dengue structural proteins and nonstructural protein NS1 expressed by baculovirus recombinant
5 induces resistance to dengue virus encephalitis. *J. Virol.* 62 p.3027).

The small protein M (8 kDa) is synthesized like a glycosylated precursor named pre-M (22 kDa approximately), which suffers a late endoproteolytic cleavage just before or
10 after the liberation of the virus of the infected cell (Murray, J. M. et al. 1993. Processing of the dengue virus type 2 proteins prM and C-prM. *J. Gen. Virol.* 74 p.175). The cleavage, which is probably done by a cellular protease, seems to happen in the post-Golgi acidic vesicles, being
15 inhibited by agents that destabilized the low pH of this vesicles (Randolph, V. B. et al. 1990. Acidotropic amines inhibit proteolytic processing of Flavivirus prM protein. *Virol.* 174 p.450). The fragment pre- has been identified in vitro only in the extracellular medium, its destiny in vivo
20 remains unknown (Murray, J. M. et al. 1993. Processing of the dengue virus type 2 proteins prM and C-prM. *J. Gen. Virol.* 74 p.175).

It is thought that the function of pre-M/M during the Flavivirus exocytic via is to avoid the activation of the
25 fusogenic membrane domain of E with the acidic pH of the environment (Randolph, V. B. et al. 1990. Acidotropic amines inhibit proteolytic processing of Flavivirus prM protein. *Virol.* 174 p.450); if this event happens, then the viral liberation will be prevented. In fact, it has been determined
30 that pre-M and E interact in the immature intracellular virions (Wengler, G. y Wengler, G. 1989. Cell-associated West Nile flavivirus is covered with E+pre-M protein heterodimers which are destroyed and reorganized by proteolytic cleavage during virus release. *J. Virol.* 63 p.2521), and that the
35 native conformation of E it is only acquired in the presence

of pre-M (Konishi, E. y Mason, P.W. 1993. Proper maturation of the Japanese encephalitis virus envelope glycoprotein requires cosynthesis with the premembrane protein. **J. Virol.** 67 p.1672). In addition, already liberated virions that only
5 have pre-M in their membranes show, in general, a lower infectivity than the completely mature virion (Wengler, G. y Wengler, G. 1989. Cell-associated West Nile flavivirus is covered with E+pre-M protein heterodimers which are destroyed and reorganized by proteolytic cleavage during virus release.
10 **J. Virol.** 63 p.2521), in which although M and pre-M are present, the former is predominant.

Pre-M and M offer an active protection when they have been expressed in recombinant vaccinia virus, but this do not
15 happen with the fragment pre- (Bray, M. y Lai, C.-J. 1991. ~~Dengue virus pre-membrane and membrane proteins~~ elicit a protective immune response. **Virol.** 185 p.505), besides the combination pre-M or M with glycoprotein E in the same recombinant Vaccinia virus gives in general levels of
20 protection higher than those reached by each protein individually. Similarly, certain antibodies against pre-M/M are able to protect passively in mice (Kaufman, B. M. et al. 1989. Monoclonal antibodies for dengue virus prM glycoprotein protect mice against lethal dengue infection. **Am J. Trop. Med.**
25 **& Hyg.** 41 p.576).

The use of synthetic peptides has allowed to establish the molecular basis of antigenicity according to spacial conformation and the immunological properties of the antigen
30 involved—[Arnon, R. y Sola, M. 1985. ~~Synthetic Vaccines~~ : present and future. **Ann. Inst. Pasteur/Immunol** 136 D, 271-282]. The synthetic peptides as anti-dengue vaccine subunits will allow to include in the final formulation only the protective epitopes that do not cause immune-amplification
35 (Halstead, S.B., y O'Ruourke, E.J. 1977. Dengue viruses and

mononuclear phagocytes. I. Infection enhancement by non-neutralizing antibody. *J. Exp. Med.* 146 p.201; Halstead, S.B. 1979. In vivo enhancement of dengue virus infection in rhesus monkeys by passively transferred antibody. *J. Infect. Dis.* 140 p.527), or alternatively, to include protective peptides of each of the four serotypes. The characterization of the antigenic determinants of E and NS1 has been carried out successfully. However, there are no similar studies on the also important protein pre-M/M, that is why the results of this paper are a first step on that direction.

The efforts to express the flaviviral protein pre M, M and E in *E. coli* have not always been successful (Chambers, T. J. et al. 1990. Production of yellow fever virus proteins in infected cells: identification of discrete polyprotein species and analysis of cleavage kinetics using region-specific polyclonal antiserum. *Virology* 177 p.159; Yan, B.-S. et al. 1994. Truncating the putative membrane association region circumvents the difficulty of expressing hepatitis C virus protein E1 in *Escherichia coli*. *J. Virology Methods* 49 p.343). Apparently, the hydrophobic regions these protein have in C-terminal are the cause of the low or undetectable heterologous expression levels (Yan, B.-S. et al. 1994. Truncating the putative membrane association region circumvents the difficulty of expressing hepatitis C virus protein E1 in *Escherichia coli*. *J. Virology Methods* 49 p.343).

The expression of those proteins (as well as NS1) in *E. coli*, in general have been obtained by fusion (fragmented or not) to other bacterial proteins (e.g. β -galactosidase (Cane, P.A. y Gould, E.A. 1988. Reduction of yellow fever mouse neurovirulence by immunization with a bacterially synthesized non-structural protein (NS1) fragment. *J. Gen. Virology* 69 p.1241), TRPE (Megret, F. et al. 1992. Use of recombinant fusion proteins and monoclonal antibodies to define linear and

discontinuous antigenic sites on the Dengue envelope glycoprotein. **Virology** 187 p.480) and the protein A of *Staphylococcus aureus* (Murray, J. M. et al. 1993. Processing of the dengue virus type 2 proteins prM and C-prM. **J. Gen. Virol.** 74 p.175). In these fusion proteins most of the relevant conformational epitopes are absent, because though the antisera generated against them can recognize the whole virus, they are not able neither to neutralize it nor to inhibit their hemagglutinating properties (Megret, F. et al. 1992. Use of recombinant fusion proteins and monoclonal antibodies to define linear and discontinuous antigenic sites on the Dengue envelope glycoprotein. **Virology** 187 p.480). However, recent reports show that the solubility of the fusion proteins, and as a consequence, the use of non-denaturalizing methods for its purification, may preserve most of the neutralizing (Seif, S.A. et al. 1995. Finer mapping of neutralizing epitope(s) on the C-terminal of Japanese encephalitis virus E-protein expressed in recombinant *Escherichia coli* system. **Vaccine** 13 p.1515) and protective (Srivastava, A.K. et al. 1995. Mice immunized with a dengue type 2 virus E and NS1 fusion protein made in *Escherichia coli* are protected against lethal dengue virus infection. **Vaccine** 13 p.1251) epitopes they possess.

In the case of pre-M/M, its pre-domain has 6 cysteins involved in 3 disulfide bridges, as well as an N-glycosylation site in the asparagine 69. The structure of E and NS1 is even more complicated; it involves 6 disulfide bridges and several N-glycosylation sites. However, the little ectodomain of M is apparently free of those conformational complexities because it does not have cysteins, and it is not glycosylated in its natural form.

The insertion of heterologous fragments in permissive areas of immunogenic-proteins which topology is more or less known

and immunization of these fusions is a complementary alternative to the use of synthetic peptides. Both strategies allow defining the presence of sequential B cell, as well as T cell epitopes. The biological importance of these epitopes could be experimentally evaluated to decide where to include them or not in certain vaccine preparation.

Detailed description of the invention.

A number of five peptides from Pre-M/M protein of Dengue 2 virus which covers the 58 % of the aminoacid sequence (97/166 AA) were chemically synthesized. They were 3-31; 45-67; 57-92; 69-93; and 103-124, which were subsequently named B 19-6; B 20-2; B 19-5; B 20-1; B 20-3 respectively.

Peptides were inoculated in Balb/c mice both conjugated or not to a carrier protein. The sera obtained after immunization with the conjugated peptides were tested by in vitro neutralization by reducing the number of plaques and by ELISA. We also studied the active protection against a Dengue 2 viral challenge in the immunized mice.

In the case of mice immunized with the non-conjugated peptides, the antibody response was evaluated by ELISA and the proliferative response of spleen T lymphocytes against Dengue 2 virus was evaluated too.

Fusion proteins were also obtained, and two of the four regions covered by peptides (1-42 and 92-133) were inserted to them and were expressed in E.coli bacteria. Immunization with these fusions will complement the results obtained with the synthetic peptides.

The presence of B cell epitopes in both mice and humans was demonstrated as the peptides were recognized by antibodies from the immunized mice and by sera from patients who had the clinical and serological diagnosis of Dengue virus, using ELISA in both cases. Peptides 19-6 and 20-3 were able to induce neutralizing antibody production against the four Dengue virus serotypes.

Virus-specific proliferative responses were demonstrated in mice immunized with non-conjugated peptides 19-6 and 19-5.

Mice immunized with conjugated peptides 19-6, 20-1, and 19-5 showed an statistically significant level of protection when they were challenged with Dengue 2 virus.

Thus, the presence of sequential epitopes in Pre-M/M protein of Dengue virus 2 was demonstrated, as well as their relevance in the immune response against these flaviviruses.

EXAMPLES OF REALIZATION

EXAMPLE 1. Prediction of antigenic regions and of T-cells epitopes of pre-M/M protein of Dengue virus.

Different theoretical methods were applied to predict the antigenic regions in the pre-M/M protein of D2 virus. These regions are those more likely to be recognized by antibodies obtained against the viral proteins, as well as to generate antibodies that recognized the original proteins. Some methods to predict T-cell epitopes were applied.

Five initial peptides that have possible B- and T- cell epitopes were found (4 in pre- and 1 in M). The study of the antigenic structure of these proteins and the experimental determination of possible immunologically important peptides was based on this finding.

1.1 Predictions of humoral antigenicity.

Methods used to predict the antigenicity were based on the aminoacidic sequence, since neither the tridimensional structure of the pre-M/M protein of Dengue virus has been determined experimentally nor there is a significant similarity at sequence level with any protein of known tridimensional structure.

The A 15 strain of Dengue 2 isolated in Cuba in 1981 (Kourí, G. et al. 1986. Hemorrhagic dengue in Cuba: history of an epidemic. *Bull. P.A.H.O* 20 p.24) was used to accomplish this example. The potentially antigenic regions were selected according to the following criteria:

a) regions of high antigenic propensity according to different prediction methods based on hydrophilicity (Hoop, T.P. y Woods, K.R. 1981. Prediction of protein antigenic

determinants from amino acid sequences. **Proc. Natl. Acad. Sci. USA** 78 p.3824; Parker, J.M.R. et al. 1986. New hydrophilicity scale derived from HPLC peptide retention data: correlation of predicted surface residues with antigenicity and X-ray derived accessible sites. **Biochemistry** 25 p.5425), flexibility (Karplus, P.A. y Schultz, G.E. 1985. Prediction of chain flexibility in proteins. A tool for the selection of peptide antigens. **Naturwissenschaften** 72 p.212) and accessibility (Emini, E.A. et al. 1985. Induction of hepatitis A virus-neutralizing antibody by a virus specific synthetic peptide. **J. Virol.** 55 p.836).

b) regions with high possibility of forming loops and turns according to predictions of secondary structure that use PHD (Rost, B. y Sander, C. 1993. Prediction of protein secondary structure at better than 70% accuracy. **J. Mol. Biol.** 232 p.584; Rost, B. y Sander, C. 1994. Combining evolutionary information and neural networks to predict protein secondary structure. **Proteins** 19 p.55; Rost, B. y Sander, C. 1994. Conservation and prediction of solvent accessibility in protein families. **Proteins** 20 p.216).

c) regions of high variability that include or not insertions/suppressions in respect to other flaviviruses, as well as potential regions of glycosylation in other flaviviruses that are used or not in Dengue virus.

25 a) Antigenicity profiles.

Figure 1 shows the profiles that are obtained when applying to the pre- and M segments 4 properties of the amino acids related to the antigenicity.

In the pre- region there are high hydrophilicity and accessibility values in the regions that have the residues 6-9, 16-21, 28-31, 42-47, 58-65 and 82-91. It is remarkable the existence of a vast hydrophobic region between the residues 41-76, which corresponds to the transmembrane helices that are thought not to be exposed to the immune system. In the little ectodomain of M (residues 1-40) the region of major

hydrophilicity/accessibility goes between 13-31 amino acids, especially at its beginning (AA 13-16).

b) Predictions of secondary structure.

Figure 2 shows the predictions of secondary structure and
5 accessibility of the pre- and M segments according to PHD
program. The results of the predictions show that many
potentially antigenic regions (according to the profiles of
Figure 1) are predisposed to form loops/ β -turns with exposed
residues in the surface of the protein. It is predicted the
10 formation of transmembrane helices for the region between
aminoacids 41-76 of protein M, and this matches with the
hydrophobic character of this region and suggests that the
antigenic peptides of M are mainly in the ectodomain (1-40).

**c) Alignments of sequences of protein pre- and M of Dengue
15 and other Flavivirus. Variability and glycosylation.**

In general, regions that are not exposed to the solvent have
a bigger conservation in the family of homologous proteins.
Therefore, regions of higher variability have a higher
probability to be exposed.

20 In the case of viruses, variability is also an escape
mechanism for the immunological pressure; of course this does
not exclude that some conserved regions might be antigenic or
that there could be conserved regions in the surface.

The analysis of sequences of regions pre- and M of 15
25 isolates of the 4 serotypes of Dengue virus shows that at
least the 69% of the residues are strictly conserved. The
more important variable residues are in the positions 28-30,
55-59, 69-72 and 80-83 of pre-, as well as in 27-30 of M. In
general; these zones match the maximum of antigenic profiles
30 of Figure 1.

The comparison of the sequences of these regions in more than
30 flaviviral isolates shows that the region 1-33 of pre is
highly variable, with possible loops predisposed to
insertions/suppressions (in the positions 8 and 30) and
35 several potential sites of N-glycosylation. On the contrary,

the variability is lower in the domain 33-91 of pre-; there are several positions strictly conserved in all flaviviruses, for example: 6 cysteins forming of 3 disulfide bridges, at least 5 acid residues in the region 40-65, as well as the
 5 basic sequence 87-91, after which the endoproteolytic cleavage occurs just before or during the liberation of the mature virus. (Figure 3)

Asn-69, conserved residue in the antigenic Dengue complex has the only N-glycosylation of pre-M/M protein of the complex.
 10 However, in the Flaviviridae family this region is in a possible exposed loop of high variability. At the same time the pre-M/M residues of Dengue virus that match the potential N-glycosylation sites in other Flavivirus (for example, AA 14 in JE, SLE, MVE YF and AA 32 in LI, LAN, YF TBE) are β -turns
 15 close to zones considered as antigenic.

1.2 Prediction of T cell epitopes.

The prediction was done by two independent methods: the Rothbard and Taylor pattern method (Rothbard, J.B. y Taylor, W.R. 1988. A sequence pattern common to T-cell epitopes. **EMBO**
 20 **J.** 7 p.93.) and the determination of fragments with propensity to form alpha-helix structures (AMPHI 7 and 11) (Margalit, H. et al. 1987. Prediction of immunodominant helper T cell antigenic sites from the primary sequence. **J. Immunol.** 138 p.2213.). Results are shown in Figure 4.

25 1.3 Peptides proposed for the identification of relevant epitopes.

The determination of neutralizing and protective peptides in general is very important for the development of more efficient vaccines, and peptides from regions of high
 30 antigenic propension are very useful for their identification; especially of those of linear nature.

Table 1 shows a set of peptides that include regions predisposed to have B and T cell epitopes (according to the several predictive methods used in this example) of pre-M/M
 35 protein of D2 virus. If the validity of that prediction is

demonstrated experimentally, the immunological important epitopes of each region will be placed accurately by the design of low size peptides in each of them.

5. **Table 1.** Antigenic peptides proposed in pre-M/M protein of Dengue virus.

CODE	SEQUENCE	REGION
B 19-6	LTTRNGEPHMIVMROEKGSLLFKTGDTV	3-31
B 20-2	CEDTITYKCPLLQNEPEDIDCW	45-67
B 19-5	RQNEPEDIDCWCNSTSTWVTYGTCTTTGHRREKRS	57-92
B 20-1	NSTSTWVTYGTCTTTGHRREKRSV	69-93
B 20-3	LETRTETWMSSEGAWKHAQRIE	103-124

- 10 **EXAMPLE 2. Chemical synthesis of oligopeptides and oligonucleotides.**

2.1. Synthesis of oligopeptides.

All peptides were synthesized using a Boc- strategy in solid phase on the p-methyl-benzhydrylamine resin (resin MBHA,
15 BACHEM, Switzerland).

The protected amino acids were offered by BACHEM. The protection of reactive groups of the amino acid chain was: Arg (Tos), Asp (OBzl), Cys (4-Me-Bzl), Glu (OBzl), Lys (2-Cl-Z), Trp (CHO), Tyr (Cl₂-Bzl), Thr (Bzl). Asn, Gln and Pro
20 were used without protection in the lateral chains.

Discarding of the Boc-amino protective group was carried out using 37.5% trifluoroacetic acid in dichloromethane. Activation with diisopropyl-carbodiimide (DIC) in situ was used for the coupling reaction of each residue, except for
25 amino acids Asn and Gln, which were activated using DIC and 1- hydroxybenzotriazole in N,N-dimethylformide.

Final desprotection and peptide liberation of the resin was accomplished in special equipment. The procedure used is known as Low-High HF.

30 During the first part of the procedure (Low HF), the protected-resin system was treated with HF (25%): DMS (65%): p-cresol (10%) during 120 minutes at 0°. The mixture was

replaced by HF (25%): DMS (60%): EDT (10%): p-cresol (5%) in the case of Trp-containing peptides. Subsequently, the resin-peptide was washed several times with diethyl ether, dichloromethane and 2-propanol, and was vacuum-dried.

- 5 During the second part of the procedure (High HF), the resin-peptide was treated with HF (90%): anizole (10%) during 60 minutes at 0°.

The raw product was washed with ether, then was extracted with 30% acetic in water and finally was lyophilized.

- 10 Peptides were characterized by RP-HPLC in a BAKER C-18 (4.6 x 100 mm) column and by spectrometry of masses using FAB as ionization method in a JEOL HX- 110 HF equipment.

The aminoacidic sequence as well as its location in the pre-M/M protein of Dengue virus is shown in Table 1.

- 15 **2.2. Synthesis of oligonucleotides.**

Oligonucleotides were automatically synthesized in the equipment Gene Assembler Plus, according to the phosphoramidite method.

The sequence of the six oligonucleotides is shown in Table 2.

20

Table 2. Sites Xba I and HcoR I that are created in each end for its later manipulation are underlined and double-underlined, respectively. The reading frame of the codified protein is defined by the triplets of bases.

25

Oligonucleotide position	Nucleotidic sequence
1) 5' pre DEN-2	5'-TTT CTA GAT TTC CAT TTA ACC ACA CGT T-3'
2) 3' pre DEN-2	5'-T TTC TAG ACC AAG GTC CAT GGC CAT GAG-3'
3) 5' M DEN-2	5'-TTT CTA GAA TCA GTG GCA CTC GTT CCA CAT G-3'
4) 3' M DEN-2	5'-T TTC TAG AAA GCC TGG ATG TCT CAA GAT CCA-3'
5) 5' M DEN-4	5'-TTT CTA GAT TCA GTA GCT TTA ACA CCA C-3'
6) 3' M DEN-4	5'-T <u>TGA</u> ATT CGC GAA TCT TGG GTT TCT GAG-3'

EXAMPLE 3. Coupling of peptides to a carrier protein and immunization scheme.

3.1 Coupling of peptides to BSA.

- 30 The coupling of peptides was performed as follows:

1. Activation of BSA: Drop by drop and with shaking, 80 μ l of the bifunctional reagent m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) 5 μ g/ μ l in dimethylformamide, were added to a dissolution of 2.8 mg of bovine albumin fraction V (BSA) in 250 μ l of PBS. Then, it was kept in agitation at room temperature during 30 minutes, and the mixture was passed through a PD10 column.
2. Coupling of the peptide to the activated BSA: a dissolution of 1 mg of peptide dissolved in 300 μ l of PBS was added to the activated BSA solution drop by drop and with shaking. It was kept at room temperature during 3 hours, and concentration was determined by the Lowry method.

3.2 Immunization scheme

- 15 The immunization scheme of peptides linked to BSA is the following:

Male Balb/c mice of 4-6 weeks old were immunized with 50 μ g of the peptide-BSA conjugate intraperitoneally. In addition, two immunization schemes were performed, one with BSA and the other with PBS. A total of 4 inoculations were carried out, each of them 15 days apart. The Freund's Complete Adjuvant was used in the first doses, and the Freund's Incomplete Adjuvant in the others. A blood sample was extracted from the retro orbital vein seven days after the last inoculation.

- 25 The obtained sera of each scheme were placed at -20°C to be used later.

EXAMPLE 4. In vitro plaque-reduction neutralization test.

Neutralization technique was carried out according to Morens (Morens, D.M. et al. 1985. Simplified plaque reduction neutralization assay for dengue viruses by semimicro methods in BHK-21 cells: Comparison of the BHK suspension test with standard plaque reduction neutralization. *J. Clin. Microbiol.* 22 p.250).

Dilutions of anti-peptide sera and of anti-BSA controls and

negative sera from 1/10 to 1/640 were prepared. Each dilution of sera was put into contact with a dilution of the virus (strain A 15 of Dengue 2) having 15-20 PFU/50 μ l.

The mixture was incubated at 37°C during 1 hour. A total of 50 μ l of each mixture were added in triplicate to BHK-21 cells in plates of 24 wells, and they were incubated in a CO₂ incubator at 37°C during 4 hours. Then, 0.5 ml of carboxymethylcellulose-containing medium were added, and it was incubated again for several days considering the viral serotype used. After these days tinction and the count of lysis plaques produced by the virus were carried out. Titer was expressed in each case as the dilution at which it was obtained the 50 % of plaque number reduction. The results are shown in Table 3.

15

Table 3. PRNT of the anti-peptide sera against 19-6 and 20-3.

Anti-peptide	Neutralizing titer for each serotype			
	D ₁	D ₂	D ₃	D ₄
B 19-6	1/100	1/180	1/60	1/160
B 20-3	1/110	1/80	1/80	1/80

20 EXAMPLE 5. Identification of T cell epitopes.

The presence of T cell epitopes in the peptides of pre-M was evaluated throughout the study of anti-peptide antibody response elicited in free peptides (non-conjugated) immunized mice. Primed animals demonstrated higher serum antibody production in response to a booster dose of antigen when compared to the response in naive animals. These results confirm the existence of B cell epitopes in these peptides and show that these sequences contains T cell epitopes, which are able to stimulate Th activity *in vivo* to improve the titers of antibody response.

30

Virus-specific proliferative responses of spleen T

lymphocytes were demonstrated in peptide immunized BALB/c mice. T cells from 19-6 and 19-5 immunized mice proliferated in an *in vitro* blastogenesis assay when they were cultured with the dengue 2 virus. However, the 20-2 peptide did not
5 elicit a significant proliferative response against the virus. It could contain a T cell cryptic epitope, being recognized in the free form of peptide but not like a result of the immunodominant epitopes presentation and processing of the virus in a natural infection.

10 **EXAMPLE 6. Protection assay.**

Mice were challenged 7 days after the last immunization by intracranial injection with an dilution of 1/2500 (corresponding to 100 LD50 lethal doses) of live, mouse-adapted dengue-2 virus (strain A15). Mice were observed for
15 up to 21 days for morbidity and mortality. Data were tested for statistical significance using Fisher's test. The percent survival in peptide immunized and control animals are shown in Figure 6. The level of protection induced for the peptides 19-5, 19-6 and 20-1 was statistically significant ($p < 0,05$).

20 **EXAMPLE 7. Indirect ELISA to detect anti-peptide antibodies.**

Human sera:

Peptides 19-6, 20-1, 20-2, 20-3 were fixed to the plates in a 10 µg/ml concentration in coating buffer, then they were incubated at 4°C overnight. Sera were added diluted 1/200 in
25 PBS-Tween 20. Finally, total human /peroxidase anti-immunoglobuline conjugate was added, and subsequently, the substrate (orthophenylendiamine, H_2O_2 , 0.05 M phosphate citrate buffer, pH 5) was added. The lecture was carried out in an ELISA reader at 492 nm and the cut-off value for each
30 peptide was determined.

The sera used were from subjects having viral clinical infection that was serologically diagnosed as Dengue by the techniques of Inhibition of the hemagglutination (Clarke, D.H. y Casals, J. 1958. Techniques for hemagglutination and
35 hemagglutination - inhibition with Arthropod Borne Virus. *Am.*

J. Trop. Med. Hyg. 7 p.561) and ELISA of inhibition (Vázquez, S. , Fernández, R. 1989. Utilización de un método de ELISA de Inhibición en el diagnóstico serológico de dengue. **Rev. Cub. Med. Trop.** 41(1) p18-26) for total anti-dengue antibodies.

- 5 The study included 118 sera from patients of the epidemics occurred in Cuba 1981, Panama 1994 and Costa Rica 1994. Dengue virus 2 was isolated in these epidemics, besides serotype 1 and 4 in Costa Rica; they were classified according to the titers of inhibiting antibodies of
10 hemagglutination in cases of primary and secondary infections.

The 46.6% of the sera were positive to the 4 peptides used. Percentages of positivity of 56.8%, 79.6%, 77.1% and 83.1% to peptides B 19-6, 20-1, 20-2, and 20-3 were obtained
15 respectively.

The average of the reactivity index, calculated by the optical density quotient of the sample/cut-off value, for each peptide was 1.07, 1.52, 1.57 and 1.49 for each peptide.

Mice sera:

- 20 The indirect ELISA used was as described above but using an anti-mouse Ig conjugated to peroxidase. Antibody titers obtained in the anti-peptides sera were generally above 1/10 000.

EXAMPLE 8. Insertion of pre-M/M fragments in the P64k protein of *Neisseria meningitidis*.

- In this example we expressed fragments of the pre M/M protein of Dengue 2 (A 15 strain) and of Dengue 4 (814669 strain) (Zhao, B. et al. 1986. Cloning full-length dengue type 4 viral DNA sequences: analysis of genes coding for structural
30 proteins. **Virology** 155 p.77) inserted in a *N. meningitidis* protein previously characterized in our group (Silva, R. et al. 1992. Nucleotide sequence coding for an outer membrane protein from *Neisseria meningitidis* and use of said protein in vaccine preparations. **European Patent 0 474 313**, 1997): P64k,
35 which have demonstrated to be highly immunogenic in several

animal models. Besides, the level of expression of P64k in *E. coli* reaches more than 30% of the total protein of the bacteria.

P64k protein (64 kDa) of dimeric nature, has two functional domains in each subunit: one with lipoic acid-binding activity (1-100) and the other with lipoamide-dehydrogenase activity (117-594). Both have been identified as conformational domains relatively independent by crystallography of X-rays (Li de la Sierra, I. et al. 1994. Crystallization and preliminary X-ray investigation of a recombinant outer membrane protein from *Neisseria meningitidis*. *J. Mol. Biol.* 235 p.1154; Li de la Sierra, I. et al. 1997. Molecular structure of the lipoamide dehydrogenase domain of a surface antigen from *Neisseria meningitidis*. *J. Mol. Biol.* 269 p.129).

The former was selected (in the aminoacidic position 45) to perform the insertions of fragments 1-42 and 92-133 of pre-M/M, because this little domain is more exposed and does not seem to be involved in dimer-formation. This suggested that the global structure of the chimeric protein with respect to the natural P64k would be less altered than if an insertion site were made in the domain 117-594, which, in addition participates directly in the formation of the dimer.

The region coding for amino acids 44-53 (TLETDRKATMD), which include the region of lipoic acid binding of the P64k gene used in the production of fusion proteins, was preliminary changed to TLDLEMD. This modification was carried out to avoid the recognition of P64k by the sera of patients having primary cirrhosis, who have self-antibodies against homologous epitopes present in the human dehydrolipoamide acetyltransferase mitochondrial (Tuailon, N. et al. 1992. A lipoyl synthetic octadecapeptide of dihydrolipoamide acetyltransferase specifically recognized by anti-M2 autoantibodies in primary billiard cirrhosis. *J. Immunol.* 148 p.445).

The strategy to produce the two clones is explained below:

Fragments Pre-2, M-2 and M-4 were amplified by Polymerase Chain Reaction, using the combination of oligonucleotides 1 and 2, 3 and 4, and 5 and 6, respectively (see Table 2), and using pD-5 plasmid as template. This pD-5 plasmid includes a copy of preM/M gene from Dengue-2 virus (strain A-15) cloned into pBluescript vector (stratagene). DNA bands obtained in each case (120 bp) were digested with Xba I (Pre-2 and M-2) or Xba I/EcoR I (M-4), and they were cloned into the corresponding sites that were artificially created in position 135-145 of P64k gene, cloned into vector pM-92. Besides, a chimeric clone that include M-2 and M-4 bands in the already mentioned sites Xba I and EcoR I was generated by triple ligation. Recombinant clones bearing the inserts in the right orientation were identified by restriction analysis and DNA-sequencing.

The fusion proteins generated by clones of Pre-2 (pD31), M-2 (pD30), M-2/M-4 (pD33), and M-4 (pD34) were expressed under the promoter of the tryptophan operon (ptrp) in the E. coli strain MM294 (F^- end A1 hsdR17 ($r_k^- m_k^+$) sup E44 thi-1 relA1? RfbD1? SpoT1?). All were obtained in the expected sizes and with levels of expression up to 30% of the total proteins of the bacteria, though PD31 protein showed a high instability (Figure 7). All fusion proteins were recognized by some mouse monoclonal antibodies anti-P64k in ELISA (data not shown) and Western blotting (Figure 8), where a remarkable degradation in the whole-cell extract was detected. The aminoacidic sequence of these proteins is shown the Sequence List.

Mouse immunization with PD33 and PD34 fusion proteins semipurified by a non-denaturalizing protocol, has elicited high titers against them in ELISA (up to 1/100 000), and at the same time antibodies with titers up to 1/4000 in ELISA against the synthetic peptides have been obtained.

DESCRIPTION OF THE FIGURES.

Figure 1. Hydrophyllicity, accessibility and flexibility profiles of pre- (A) and M (B) proteins of Dengue virus.

5 **Figure 2.** Prediction of the secondary structure and of accessibility of pre (A) and M (protein). AA: amino acids. PHD sec: prediction of secondary structure (E= beta, H= helix, L= loop) P- 3 acc. Prediction of accessibility (e=exposed, b= non-exposed). Sub sec (Sub acc) residues in
10 which the prediction of secondary structure (accessibility) is 82.4% (70%) efficacious.

Figure 3. Variability profiles of pre- and M proteins. Variability was calculated considering 3 sets of Flavivirus sequences. Dengue: sequences of 15 Dengue isolations. MBV:
15 Flavivirus sequences transmitted by mosquitoes that include Dengue virus, Kunji, West Nile Virus, Murray Valley Encephalitis and Saint Louis Encephalitis. Flavivirus: sequences of more than 30 different flaviviral isolations: (MBV + Yellow fever, Langat, Louping Ill and Tick-Borne
20 Encephalitis).

Figure 4. Prediction of T cell epitopes of pre (A) and M (B) proteins: AMPHI 7 (11): prediction of amphipatic segments of 7 (11) residues, positive residues are the central amino acids of an amphipatic block potentially antigenic. RT 4 (5):
25 prediction of antigenic profiles of 4 (5) residues, positive residues are those who fulfill the profiles.

Figure 5. Proliferative response to dengue 2 virus antigens (at concentrations of 10, 20 and 40 µg/ml) of spleen T cells from peptides-immunized mice 19-6, 19-5 and 20-2.

30 **Figure 6.** Percent survival in peptide immunized and control animals. The level of protection induced for the peptides 19-5, 19-6 and 20-1 was statistically significant.

Figure 7. 10% SDS-PAGE of MM294 *E. coli* strain transformed with fusion proteins and P64k protein (pM-92 plasmid). Lanes:
35 1- Untransformed MM294 strain, 2- pM-92/MM294, 3- pD-

30/MM294, 4- pD-31/MM294, 5- pD-33/MM294, 6- pD-34/MM294.

Figure 8. Western blot using AcM 114 of MM294 *E. coli* strain transformed with fusion proteins and P64k protein (pM-92 plasmid). Lanes: 1- Untransformed MM294 strain, 2- pM-92/MM294, 3- pD-30/MM294, 4- pD-31/MM294, 5- pD-33/MM294, 6- pD-34/MM294.

SEQUENCE LISTING

10

(1) GENERAL INFORMATION:

(i) APPLICANT:

15

(A) NAME: CENTRO DE INGENIERIA GENETICA Y BIOTECNOLOGIA

(B) STREET: Ave. 31 entre 158 y 190, Playa

(C) CITY: Ciudad de La Habana

(E) COUNTRY: Cuba

(F) POSTAL CODE (ZIP): 10600

(G) TELEPHONE: 53 7 218466

20

(H) TELEFAX: 53 7 218070 / 336008

(A) NAME: INSTITUTO DE MEDICINA TROPICAL "PEDRO KOURI"

(B) STREET: Autopista Novia del Mediodia Km 6, La Lisa

(C) CITY: Ciudad de La Habana

25

(E) COUNTRY: Cuba

(F) POSTAL CODE (ZIP): 11100

(G) TELEPHONE: 53 7 220633

(H) TELEFAX: 53 7 335061

30

(ii) TITLE OF INVENTION: PRE-M/M PROTEIN EPITOPES OF THE DENGUE VIRUS, SYNTHETIC PEPTIDES AND ITS USE.

(iii) NUMBER OF SEQUENCES: 9

35

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

40

(vi) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: CU 13/97

(B) FILING DATE: 15-JAN-1997

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 29 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

10 (iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

- 15 (A) ORGANISM: Dengue virus
(B) STRAIN: Dengue-2
(C) INDIVIDUAL ISOLATE: A-15

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Leu Thr Thr Arg Asn Gly Glu Pro His Met Ile Val Met Arg Gln Glu
1 5 10 15
25 Lys Gly Lys Ser Leu Leu Phe Lys Thr Gly Asp Gly Val
20 25

(2) INFORMATION FOR SEQ ID NO: 2:

30

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
35 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: NO

40 (v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Dengue virus
(B) STRAIN: Dengue-2
45 (C) INDIVIDUAL ISOLATE: A-15

22

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Cys Glu Asp Thr Ile Thr Tyr Lys Cys Pro Leu Leu Arg Gln Asn Glu
 1 5 10 15
 Pro Glu Asp Ile Asp Cys Trp
 20

10 (2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 36 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS:
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Dengue virus
 (B) STRAIN: Dengue-2
 (C) INDIVIDUAL ISOLATE: A-15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Arg Gln Asn Glu Pro Glu Asp Ile Asp Cys Trp Cys Asn Ser Thr Ser
 1 5 10 15
 Thr Trp Val Thr Tyr Gly Thr Cys Thr Thr Thr Gly Glu His Arg Arg
 20 25 30
 Glu Lys Arg Ser
 35

40 (2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS:
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(A) ORGANISM: Dengue virus
(B) STRAIN: Dengue-2
(C) INDIVIDUAL ISOLATE: A-15

Asn Ser Thr Ser Thr Trp Val Thr Tyr Gly Thr Cys Thr Thr Thr Gly
1 5 10 15

Glu His Arg Arg Glu Lys Arg Ser Val
20 25

(A) LENGTH: 22 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(v) FRAGMENT TYPE: internal

(A) ORGANISM: Dengue virus
(B) STRAIN: Dengue-2
(C) INDIVIDUAL ISOLATE: A-15

Leu Glu Thr Arg Thr Glu Thr Trp Met Ser Ser Glu Gly Ala Trp Lys
1 5 10 15
His Ala Gln Arg Ile Glu
20

(A) LENGTH: 635 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: NO

5 (vi) ORIGINAL SOURCE:

(A) ORGANISM: Fusion protein

(vii) IMMEDIATE SOURCE:

(B) CLONE: PD31

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

15 Met Ala Leu Val Glu Leu Lys Val Pro Asp Ile Gly Gly His Glu Asn
 1 5 10 15
 Val Asp Ile Ile Ala Val Glu Val Asn Val Gly Asp Thr Ile Ala Val
 20 20 25 30
 20 Asp Asp Thr Leu Ile Thr Leu Asp Leu Asp Phe His Leu Thr Thr Arg
 35 40 45
 Asn Gly Glu Pro His Met Ile Val Ser Arg Gln Glu Lys Gly Lys Ser
 50 55 60
 25 Leu Leu Phe Lys Thr Gly Asp Gly Val Asn Met Cys Thr Leu Met Ala
 65 70 75 80
 30 Met Asp Leu Gly Leu Glu Met Asp Val Pro Ala Glu Val Ala Gly Val
 85 90 95
 Val Lys Glu Val Lys Val Lys Val Gly Asp Lys Ile Ser Glu Gly Gly
 100 105 110
 35 Leu Ile Val Val Val Glu Ala Glu Gly Thr Ala Ala Ala Pro Lys Ala
 115 120 125
 Glu Ala Ala Ala Ala Pro Ala Gln Glu Ala Pro Lys Ala Ala Ala Pro
 130 135 140
 40 Ala Pro Gln Ala Ala Gln Phe Gly Gly Ser Ala Asp Ala Glu Tyr Asp
 145 150 155 160
 45 Val Val Val Leu Gly Gly Gly Pro Gly Gly Tyr Ser Ala Ala Phe Ala
 165 170 175
 Ala Ala Asp Glu Gly Leu Lys Val Ala Ile Val Glu Arg Tyr Lys Thr
 180 185 190
 50 Leu Gly Gly Val Cys Leu Asn Val Gly Cys Ile Pro Ser Lys Ala Leu
 195 200 205
 Leu His Asn Ala Ala Val Ile Asp Glu Val Arg His Leu Ala Ala Asn
 210 215 220
 55 Gly Ile Lys Tyr Pro Glu Pro Glu Leu Asp Ile Asp Met Leu Arg Ala
 225 230 235 240
 60 Tyr Lys Asp Gly Val Val Ser Arg Leu Thr Gly Gly Leu Ala Gly Met
 245 250 255

	Ala Lys Ser Arg Lys Val Asp Val Ile Gln Gly Asp Gly Gln Phe Leu	260	265	270
5	Asp Pro His His Leu Glu Val Ser Leu Thr Ala Gly Asp Ala Tyr Glu	275	280	285
	Gln Ala Ala Pro Thr Gly Glu Lys Lys Ile Val Ala Phe Lys Asn Cys	290	295	300
10	Ile Ile Ala Ala Gly Ser Arg Val Thr Lys Leu Pro Phe Ile Pro Glu	305	310	315
	Asp Pro Arg Ile Ile Asp Ser Ser Gly Ala Leu Ala Leu Lys Glu Val	325	330	335
15	Pro Gly Lys Leu Leu Ile Ile Gly Gly Gly Ile Ile Gly Leu Glu Met	340	345	350
	Gly Thr Val Tyr Ser Thr Leu Gly Ser Arg Leu Asp Val Val Glu Met	355	360	365
20	Met Asp Gly Leu Met Gln Gly Ala Asp Arg Asp Leu Val Lys Val Trp	370	375	380
	Gln Lys Gln Asn Glu Tyr Arg Phe Asp Asn Ile Met Val Asn Thr Lys	385	390	395
25	Thr Val Ala Val Glu Pro Lys Glu Asp Gly Val Tyr Val Thr Phe Glu	405	410	415
30	Gly Ala Asn Ala Pro Lys Glu Pro Gln Arg Tyr Asp Ala Val Leu Val	420	425	430
	Ala Ala Gly Arg Ala Pro Asn Gly Lys Leu Ile Ser Ala Glu Lys Ala	435	440	445
35	Gly Val Ala Val Thr Asp Arg Gly Phe Ile Glu Val Asp Lys Gln Met	450	455	460
	Arg Thr Asn Val Pro His Ile Tyr Ala Ile Gly Asp Ile Val Gly Gln	465	470	475
40	Pro Met Leu Ala His Lys Ala Val His Glu Gly His Val Ala Ala Glu	485	490	495
45	Asn Cys Ala Gly His Lys Ala Tyr Phe Asp Ala Arg Val Ile Pro Gly	500	505	510
	Val Ala Tyr Thr Ser Pro Glu Val Ala Trp Val Gly Glu Thr Glu Leu	515	520	525
50	Ser Ala Lys Ala Ser Gly Arg Lys Ile Thr Lys Ala Asn Phe Pro Trp	530	535	540
	Ala Ala Ser Gly Arg Ala Ile Ala Asn Gly Cys Asp Lys Pro Phe Thr	545	550	555
55	Lys Leu Ile Phe Asp Ala Glu Thr Gly Arg Ile Ile Gly Gly Gly Ile	565	570	575
60	Val Gly Pro Asn Gly Gly Asp Met Ile Gly Glu Val Cys Leu Ala Ile	580	585	590
	Glu Met Gly Cys Asp Ala Ala Asp Ile Gly Lys Thr Ile His Pro His	595	600	605

Pro Thr Leu Gly Glu Ser Ile Gly Met Ala Ala Glu Val Ala Leu Gly
610 615 620

5 Thr Cys Thr Asp Leu Pro Pro Gln Lys Lys Lys
625 630 635

10 (2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 635 amino acids

(B) TYPE: amino acid

15 (C) STRANDEDNESS:

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: YES

20 (iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Fusion protein

25 (vii) IMMEDIATE SOURCE:

(B) CLONE: PD30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

30 Met Ala Leu Val Glu Leu Lys Val Pro Asp Ile Gly Gly His Glu Asn
1 5 10 15
35 Val Asp Ile Ile Ala Val Glu Val Asn Val Gly Asp Thr Ile Ala Val
20 25 30
Asp Asp Thr Leu Ile Thr Leu Asp Leu Glu Ser Val Ala Leu Val Pro
35 40 45
40 His Val Gly Met Gly Leu Glu Thr Arg Thr Glu Thr Trp Met Ser Ser
50 55 60
Glu Gly Ala Trp Lys His Ala Gln Arg Ile Glu Thr Trp Ile Leu Arg
65 70 75 80
45 His Pro Gly Phe Leu Glu Met Asp Val Pro Ala Glu Val Ala Gly Val
85 90 95
50 Val Lys Glu Val Lys Val Lys Val Gly Asp Lys Ile Ser Glu Gly Gly
100 105 110
Leu Ile Val Val Val Glu Ala Glu Gly Thr Ala Ala Ala Pro Lys Ala
115 120 125
55 Glu Ala Ala Ala Ala Pro Ala Gln Glu Ala Pro Lys Ala Ala Ala Pro
130 135 140

	Ala	Pro	Gln	Ala	Ala	Gln	Phe	Gly	Gly	Ser	Ala	Asp	Ala	Glu	Tyr	Asp	
	145					150					155					160	
5	Val	Val	Val	Leu	Gly	Gly	Gly	Pro	Gly	Gly	Tyr	Ser	Ala	Ala	Phe	Ala	
					165					170					175		
	Ala	Ala	Asp	Glu	Gly	Leu	Lys	Val	Ala	Ile	Val	Glu	Arg	Tyr	Lys	Thr	
				180					185					190			
10	Leu	Gly	Gly	Val	Cys	Leu	Asn	Val	Gly	Cys	Ile	Pro	Ser	Lys	Ala	Leu	
				195				200					205				
	Leu	His	Asn	Ala	Ala	Val	Ile	Asp	Glu	Val	Arg	His	Leu	Ala	Ala	Asn	
15		210					215					220					
	Gly	Ile	Lys	Tyr	Pro	Glu	Pro	Glu	Leu	Asp	Ile	Asp	Met	Leu	Arg	Ala	
	225					230					235					240	
20	Tyr	Lys	Asp	Gly	Val	Val	Ser	Arg	Leu	Thr	Gly	Gly	Leu	Ala	Gly	Met	
					245					250					255		
	Ala	Lys	Ser	Arg	Lys	Val	Asp	Val	Ile	Gln	Gly	Asp	Gly	Gln	Phe	Leu	
				260					265					270			
25	Asp	Pro	His	His	Leu	Glu	Val	Ser	Leu	Thr	Ala	Gly	Asp	Ala	Tyr	Glu	
			275					280					285				
	Gln	Ala	Ala	Pro	Thr	Gly	Glu	Lys	Lys	Ile	Val	Ala	Phe	Lys	Asn	Cys	
30		290					295					300					
	Ile	Ile	Ala	Ala	Gly	Ser	Arg	Val	Thr	Lys	Leu	Pro	Phe	Ile	Pro	Glu	
	305					310					315					320	
35	Asp	Pro	Arg	Ile	Ile	Asp	Ser	Ser	Gly	Ala	Leu	Ala	Leu	Lys	Glu	Val	
					325					330					335		
	Pro	Gly	Lys	Leu	Leu	Ile	Ile	Gly	Gly	Gly	Ile	Ile	Gly	Leu	Glu	Met	
				340					345					350			
40	Gly	Thr	Val	Tyr	Ser	Thr	Leu	Gly	Ser	Arg	Leu	Asp	Val	Val	Glu	Met	
			355					360					365				
	Met	Asp	Gly	Leu	Met	Gln	Gly	Ala	Asp	Arg	Asp	Leu	Val	Lys	Val	Trp	
45		370					375					380					
	Gln	Lys	Gln	Asn	Glu	Tyr	Arg	Phe	Asp	Asn	Ile	Met	Val	Asn	Thr	Lys	
	385					390					395					400	
50	Thr	Val	Ala	Val	Glu	Pro	Lys	Glu	Asp	Gly	Val	Tyr	Val	Thr	Phe	Glu	
					405					410					415		
	Gly	Ala	Asn	Ala	Pro	Lys	Glu	Pro	Gln	Arg	Tyr	Asp	Ala	Val	Leu	Val	
				420					425					430			
55	Ala	Ala	Gly	Arg	Ala	Pro	Asn	Gly	Lys	Leu	Ile	Ser	Ala	Glu	Lys	Ala	
			435					440					445				
	Gly	Val	Ala	Val	Thr	Asp	Arg	Gly	Phe	Ile	Glu	Val	Asp	Lys	Gln	Met	
60		450					455					460					
	Arg	Thr	Asn	Val	Pro	His	Ile	Tyr	Ala	Ile	Gly	Asp	Ile	Val	Gly	Gln	
	465					470					475					480	

0053070218070

28

Ala His Leu Ala Val His Glu Gly His Val Ala Ala Glu

	Asp	Asp	Thr	Leu	Ile	Thr	Leu	Asp	Leu	Asp	Phe	His	Leu	Thr	Thr	Arg	
			35					40					45				
5	Asn	Gly	Glu	Pro	His	Met	Ile	Val	Ser	Arg	Gln	Glu	Lys	Gly	Lys	Ser	
		50					55					60					
	Leu	Leu	Phe	Lys	Thr	Gly	Asp	Gly	Val	Asn	Met	Cys	Thr	Leu	Met	Ala	
	65					70				75						80	
10	Met	Asp	Leu	Gly	Ser	Val	Ala	Leu	Val	Pro	His	Val	Gly	Met	Gly	Leu	
					85					90					95		
	Glu	Thr	Arg	Thr	Glu	Thr	Trp	Met	Ser	Ser	Glu	Gly	Ala	Trp	Lys	His	
15				100					105					110			
	Ala	Gln	Arg	Ile	Glu	Thr	Trp	Ile	Leu	Arg	His	Pro	Gly	Phe	Leu	Glu	
			115					120					125				
20	Met	Asp	Val	Pro	Ala	Glu	Val	Ala	Gly	Val	Val	Lys	Glu	Val	Lys	Val	
		130					135					140					
	Lys	Val	Gly	Asp	Lys	Ile	Ser	Glu	Gly	Gly	Leu	Ile	Val	Val	Val	Glu	
	145					150					155					160	
25	Ala	Glu	Gly	Thr	Ala	Ala	Ala	Pro	Lys	Ala	Glu	Ala	Ala	Ala	Ala	Pro	
				165						170						175	
	Ala	Gln	Glu	Ala	Pro	Lys	Ala	Ala	Ala	Pro	Ala	Pro	Gln	Ala	Ala	Gln	
30				180					185					190			
	Phe	Gly	Gly	Ser	Ala	Asp	Ala	Glu	Tyr	Asp	Val	Val	Val	Leu	Gly	Gly	
			195					200					205				
35	Gly	Pro	Gly	Gly	Tyr	Ser	Ala	Ala	Phe	Ala	Ala	Ala	Asp	Glu	Gly	Leu	
		210					215					220					
	Lys	Val	Ala	Ile	Val	Glu	Arg	Tyr	Lys	Thr	Leu	Gly	Gly	Val	Cys	Leu	
	225					230					235					240	
40	Asn	Val	Gly	Cys	Ile	Pro	Ser	Lys	Ala	Leu	Leu	His	Asn	Ala	Ala	Val	
				245						250					255		
	Ile	Asp	Glu	Val	Arg	His	Leu	Ala	Ala	Asn	Gly	Ile	Lys	Tyr	Pro	Glu	
45			260						265					270			
	Pro	Glu	Leu	Asp	Ile	Asp	Met	Leu	Arg	Ala	Tyr	Lys	Asp	Gly	Val	Val	
			275					280					285				
50	Ser	Arg	Leu	Thr	Gly	Gly	Leu	Ala	Gly	Met	Ala	Lys	Ser	Arg	Lys	Val	
		290					295					300					
	Asp	Val	Ile	Gln	Gly	Asp	Gly	Gln	Phe	Leu	Asp	Pro	His	His	Leu	Glu	
	305					310					315					320	
55	Val	Ser	Leu	Thr	Ala	Gly	Asp	Ala	Tyr	Glu	Gln	Ala	Ala	Pro	Thr	Gly	
				325						330					335		
	Glu	Lys	Lys	Ile	Val	Ala	Phe	Lys	Asn	Cys	Ile	Ile	Ala	Ala	Gly	Ser	
60				340					345					350			
	Arg	Val	Thr	Lys	Leu	Pro	Phe	Ile	Pro	Glu	Asp	Pro	Arg	Ile	Ile	Asp	
			355					360					365				
65	Ser	Ser	Gly	Ala	Leu	Ala	Leu	Lys	Glu	Val	Pro	Gly	Lys	Leu	Leu	Ile	
		370					375						380				

	Ile Gly Gly Gly Ile Ile Gly Leu Glu Met Gly Thr Val Tyr Ser Thr	385	390	395	400
5	Leu Gly Ser Arg Leu Asp Val Val Glu Met Met Asp Gly Leu Met Gln	405	410	415	
	Gly Ala Asp Arg Asp Leu Val Lys Val Trp Gln Lys Gln Asn Glu Tyr	420	425	430	
10	Arg Phe Asp Asn Ile Met Val Asn Thr Lys Thr Val Ala Val Glu Pro	435	440	445	
	Lys Glu Asp Gly Val Tyr Val Thr Phe Glu Gly Ala Asn Ala Pro Lys	450	455	460	
15	Glu Pro Gln Arg Tyr Asp Ala Val Leu Val Ala Ala Gly Arg Ala Pro	465	470	475	480
	Asn Gly Lys Leu Ile Ser Ala Glu Lys Ala Gly Val Ala Val Thr Asp	485	490	495	
	Arg Gly Phe Ile Glu Val Asp Lys Gln Met Arg Thr Asn Val Pro His	500	505	510	
25	Ile Tyr Ala Ile Gly Asp Ile Val Gly Gln Pro Met Leu Ala His Lys	515	520	525	
	Ala Val His Glu Gly His Val Ala Ala Glu Asn Cys Ala Gly His Lys	530	535	540	
30	Ala Tyr Phe Asp Ala Arg Val Ile Pro Gly Val Ala Tyr Thr Ser Pro	545	550	555	560
	Glu Val Ala Trp Val Gly Glu Thr Glu Leu Ser Ala Lys Ala Ser Gly	565	570	575	
	Arg Lys Ile Thr Lys Ala Asn Phe Pro Trp Ala Ala Ser Gly Arg Ala	580	585	590	
40	Ile Ala Asn Gly Cys Asp Lys Pro Phe Thr Lys Leu Ile Phe Asp Ala	595	600	605	
	Glu Thr Gly Arg Ile Ile Gly Gly Gly Ile Val Gly Pro Asn Gly Gly	610	615	620	
45	Asp Met Ile Gly Glu Val Cys Leu Ala Ile Glu Met Gly Cys Asp Ala	625	630	635	640
	Ala Asp Ile Gly Lys Thr Ile His Pro His Pro Thr Leu Gly Glu Ser	645	650	655	
	Ile Gly Met Ala Ala Glu Val Ala Leu Gly Thr Cys Thr Asp Leu Pro	660	665	670	
55	Pro Gln Lys Lys Lys	675			

60 (2) INFORMATION FOR SEQ ID NO: 9:

(1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 635 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

10 (A) ORGANISM: Fusion protein

(vii) IMMEDIATE SOURCE:

(B) CLONE: PD33

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

Met Ala Leu Val Glu Leu Lys Val Pro Asp Ile Gly Gly His Glu Asn
 1 5 10 15
 Val Asp Ile Ile Ala Val Glu Val Asn Val Gly Asp Thr Ile Ala Val
 20 25 30
 Asp Asp Thr Leu Ile Thr Leu Asp Leu Glu Ser Val Ala Leu Thr Pro
 25 35 40 45
 His Ser Gly Met Gly Leu Glu Thr Arg Ala Glu Thr Trp Met Ser Ser
 50 55 60
 Glu Gly Ala Trp Lys His Ala Gln Arg Val Glu Ser Trp Ile Leu Arg
 30 65 70 75 80
 Asn Pro Arg Phe Leu Glu Met Asp Val Pro Ala Glu Val Ala Gly Val
 35 85 90 95
 Val Lys Glu Val Lys Val Lys Val Gly Asp Lys Ile Ser Glu Gly Gly
 100 105 110
 Leu Ile Val Val Val Glu Ala Glu Gly Thr Ala Ala Ala Pro Lys Ala
 40 115 120 125
 Glu Ala Ala Ala Ala Pro Ala Gln Glu Ala Pro Lys Ala Ala Ala Pro
 130 135 140
 Ala Pro Gln Ala Ala Gln Phe Gly Gly Ser Ala Asp Ala Glu Tyr Asp
 45 145 150 155 160
 Val Val Val Leu Gly Gly Gly Pro Gly Gly Tyr Ser Ala Ala Phe Ala
 50 165 170 175
 Ala Ala Asp Glu Gly Leu Lys Val Ala Ile Val Glu Arg Tyr Lys Thr
 180 185 190
 Leu Gly Gly Val Cys Leu Asn Val Gly Cys Ile Pro Ser Lys Ala Leu
 55 195 200 205
 Leu His Asn Ala Ala Val Ile Asp Glu Val Arg His Leu Ala Ala Asn
 210 215 220

	Gly	Ile	Lys	Tyr	Pro	Glu	Pro	Glu	Leu	Asp	Ile	Asp	Met	Leu	Arg	Ala	225	230	235	240
5	Tyr	Lys	Asp	Gly	Val	Val	Ser	Arg	Leu	Thr	Gly	Gly	Leu	Ala	Gly	Met	245	250	255	
	Ala	Lys	Ser	Arg	Lys	Val	Asp	Val	Ile	Gln	Gly	Asp	Gly	Gln	Phe	Leu	260	265	270	
10	Asp	Pro	His	His	Leu	Glu	Val	Ser	Leu	Thr	Ala	Gly	Asp	Ala	Tyr	Glu	275	280	285	
	Gln	Ala	Ala	Pro	Thr	Gly	Glu	Lys	Lys	Ile	Val	Ala	Phe	Lys	Asn	Cys	290	295	300	
15	Ile	Ile	Ala	Ala	Gly	Ser	Arg	Val	Thr	Lys	Leu	Pro	Phe	Ile	Pro	Glu	305	310	315	320
	Asp	Pro	Arg	Ile	Ile	Asp	Ser	Ser	Gly	Ala	Leu	Ala	Leu	Lys	Glu	Val	325	330	335	
20	Pro	Gly	Lys	Leu	Leu	Ile	Ile	Gly	Gly	Gly	Ile	Ile	Gly	Leu	Glu	Met	340	345	350	
	Gly	Thr	Val	Tyr	Ser	Thr	Leu	Gly	Ser	Arg	Leu	Asp	Val	Val	Glu	Met	355	360	365	
25	Met	Asp	Gly	Leu	Met	Gln	Gly	Ala	Asp	Arg	Asp	Leu	Val	Lys	Val	Trp	370	375	380	
30	Gln	Lys	Gln	Asn	Glu	Tyr	Arg	Phe	Asp	Asn	Ile	Met	Val	Asn	Thr	Lys	385	390	395	400
	Thr	Val	Ala	Val	Glu	Pro	Lys	Glu	Asp	Gly	Val	Tyr	Val	Thr	Phe	Glu	405	410	415	
35	Gly	Ala	Asn	Ala	Pro	Lys	Glu	Pro	Gln	Arg	Tyr	Asp	Ala	Val	Leu	Val	420	425	430	
	Ala	Ala	Gly	Arg	Ala	Pro	Asn	Gly	Lys	Leu	Ile	Ser	Ala	Glu	Lys	Ala	435	440	445	
40	Gly	Val	Ala	Val	Thr	Asp	Arg	Gly	Phe	Ile	Glu	Val	Asp	Lys	Gln	Met	450	455	460	
45	Arg	Thr	Asn	Val	Pro	His	Ile	Tyr	Ala	Ile	Gly	Asp	Ile	Val	Gly	Gln	465	470	475	480
	Pro	Met	Leu	Ala	His	Lys	Ala	Val	His	Glu	Gly	His	Val	Ala	Ala	Glu	485	490	495	
50	Asn	Cys	Ala	Gly	His	Lys	Ala	Tyr	Phe	Asp	Ala	Arg	Val	Ile	Pro	Gly	500	505	510	
	Val	Ala	Tyr	Thr	Ser	Pro	Glu	Val	Ala	Trp	Val	Gly	Glu	Thr	Glu	Leu	515	520	525	
55	Ser	Ala	Lys	Ala	Ser	Gly	Arg	Lys	Ile	Thr	Lys	Ala	Asn	Phe	Pro	Trp	530	535	540	
60	Ala	Ala	Ser	Gly	Arg	Ala	Ile	Ala	Asn	Gly	Cys	Asp	Lys	Pro	Phe	Thr	545	550	555	560
	Lys	Leu	Ile	Phe	Asp	Ala	Glu	Thr	Gly	Arg	Ile	Ile	Gly	Gly	Gly	Ile	565	570	575	
65																				

Val Gly Pro Asn Gly Gly Asp Met Ile Gly Glu Val Cys Leu Ala Ile
580 585 590

5 Glu Met Gly Cys Asp Ala Ala Asp Ile Gly Lys Thr Ile His Pro His
595 600 605

Pro Thr Leu Gly Glu Ser Ile Gly Met Ala Ala Glu Val Ala Leu Gly
610 615 620

10 Thr Cys Thr Asp Leu Pro Pro Gln Lys Lys Lys
625 630 635

CLAIMS



1) Synthetic peptides or mimetic compounds belonging to Dengue virus pre-M/M protein, characterized for being
5 comprised between amino acid regions 3-31, 57-92, 69-93, and 103-124, and that include at least one crossreactive epitope with any of Dengue virus serotypes.

2) Synthetic peptides belonging to Dengue virus pre-M/M protein, comprising aminoacid regions 3-31, 57-92, 69-93, and
10 103-124 according to claim 1, characterized for being the described peptides in the sequence list as peptides 19-6, 19-5, 20-1 and 20-3, respectively.

3) Diagnostic tests or pharmaceutical formulations against flaviviruses characterized for including peptides or parts of
15 them, described in claims 1 and 2, conjugated or not to proteins or other carriers, independently of the adjuvant or vehicle used.

4) Flavivirus-specific antibodies or part of them, characterized because of their recognition of sequences
20 mentioned according to claims 1 and 2.

5) Vaccine or therapeutic preparations against flavivirus, or diagnostic tests, characterized for including antibodies or part of them, described in claim 4.

6) Genetic constructions characterized for including
25 epitopes of Dengue-2 and 4 virus pre-M/M protein, comprised between amino acid regions 1-42 and 92-134, fused to a carrier protein.

7) Genetic constructions according to claim 6, characterized for being fused to *Neisseria meningitidis* P64k
30 protein described in the sequence list as PD31(Pre-2), PD30(M-2), PD34(M-4), y PD33(M-2/M-4).

8) Genetic constructions according to claims 6 and 7, characterized for including at least one epitope of flaviviral pre-M/M protein.

35 9) Diagnostic tests or pharmaceutical formulations characterized for containing the product or part of it, of at

least one of the genetic constructions described according to claims 6, 7, and 8.

10) Antibodies or parts of them, characterized for recognizing the sequences mentioned according to claims 6 and
5 7.

11) Pharmaceutical formulations against ~~slavivirus~~ or diagnostic tests, characterized for containing the antibodies or part of them, described in claim 10.

