CHIMERIC CHAINS CODING FOR PROTEINS INDUCING EFFECTS AGAINST VIRUS.

Field of the Invention

The present invention is related with the field of the biotechnology and the pharmaceutical industry, in particular with the obtaining of chimeric nucleotide chains which, when introduced into an expression vector, give rise to proteins able to elicit a serotype-specific humoral immune response and protection against the Dengue virus infection; quoted from now on as DEN, avoiding the effects of the serotype-specific viral immunoamplification, causing hemorrhages and clinical complications described in this type of pathology.

Previous Technique

The Dengue virus (DEN), is a coated virus whose lipid membrane contains two of its three structural proteins: the envelope protein (E) and the membrane protein (M). The E protein covers an icosaedric nucleocapsid composed by the third of its structural proteins, the core protein. This virus belongs to the *Flaviviridae* family and four different serotypes exist. Its transmission to the man is carried out through the mosquito *Aedes aegypti* that belongs to the *Stegomia* family. The disease produced in the human for this virus was considered as benign and was described as Dengue Fever or Classical Dengue (DF) until the appearance of a more serious modality and sometimes lethal, characterized by hemorrhagic fever and shock, denominated: Hemorrhagic Dengue Fever and Dengue Shock Syndrome (HDF/DSS) (Hammon WMc. New hemorrhagic fever in children in the Philippines and Thailand. Trans Assoc Physicians 1960; 73: 140-155). Several epidemiological studies have been carried out evidencing as a risk factor the sequential infection of two different viral serotypes (Kourí GP, Guzmán MG, Brave JR. Why dengue hemorrhagic fever in Cuba) 2. An integral analysis. Trans Roy Soc Trop Med Hyg 1987; 72: 821-823). This phenomenon is explained by the immuno-enhancement, which is based on an increase of the viral infection by increasing the entrance of the virus-antibody complex to the cell through the Fc receptors of the target cell (monocytes) (halstead SB. Pathogenesis of dengue: challenges to molecular biology. Science 1988; 239: 476-481).

Different technologies have been developed to produce live attenuated vaccines, but at present there exist multiple unsolved issues on the possible benefits of these vaccines, since they could revert to the virulence, viral interference and inter-genomic recombination. Alternately, recombinant antigens can be obtained as possible components of a subunit vaccine (Feighny, R., Borrous, J. and Putnak R. Dengue type-2 virus envelope protein made using recombinant baculovirus protects mice against virus challenge. Am. J. Trop. Med. Hyg. 1994. 50(3). 322-328; Deubel, V., Staropoli, I., Megret,

F., *et al.* Affinity-purified dengue-2 virus envelope glycoprotein induces neutralizing antibodies and protective immunity in mice. Vaccine. 1997. 15, 1946-1954).

The main antigen of the virus is the envelope protein DENe. This protein is the major component of the viral surface and is thought to mediate the binding of the virus to the cellular receptor (A Heinz FX, Berge R, Tuma W et al. A topological and functional model of epitopes on the structural glycoprotein of tick-borne encephalitis virus defined by monoclonal antibodies. Virology. 1983; 126: 525). This protein has structural homology with that of the tick borne encephalitis virus (TBE) (Rey, F.A., Heinz, F.X., Mandl, C., *et al.* The envelope glycoprotein from tick borne encephalitis virus at 2 A° resolution. Nature 1995; 375: 291-298) and it is also structurally conserved among serotypes.

The insect cells constitute one of the systems most used for the expression of diverse heterologous genes that employ the baculovirus system as vectors. These vectors have been used for the expression of several combinations of structural and nonstructural proteins of the Encephalitis Japanese virus (JEV), DEN-1, DEN-2 and DEN-4, (Matsuura Y, Miyamoto M, Soto T et al. Characterization of japanese encephalitis virus envelope protein expressed by recombinant baculoviruses. Virology 1989; 173: 677-682; Deubel V, Bordier M, Megret F *et al.* Processing, secretion and immunoreactivity of carboxy terminally truncated dengue-2 envelope proteins expressed in insect cell by recombinant baculoviruses. Virology 1991; 180: 440-447; Putnak R, Feighny R, Burrous J *et al.* Dengue 1 virus envelope glycoprotein gene expressed in recombinant baculovirus elicit virus neutralization antibodies in mice and protects them from virus challenge. Am J Trop Med Hyg 1991; 45: 159-167; Feighny R, Burrous J, Putnak R. Dengue type 2 virus envelope protein made using recombinant baculovirus protects mice against virus challenge. Am J Trop Med Hyg 1994; 50: 322-328). Another system used has been the cells of *Drosophila melanogaster* expressing different variants of the E protein (PCT/US96/07627). In spite of obtaining an appropriate functional response, upon using the proteins expressed in these systems, they imply a high cost for the development of scale-up production processes; therefore, the expression in yeast has been an alternative to produce recombinant structural proteins of flavivirus. However, in the case of the DENe protein, expressed in *Pichia pastoris* (PCT/US96/07627; Sugrue R.J., Fu H., Howe J., Chan Y. Expression of the Dengue virus structural proteins in *Pichia pastoris* leads to the generation of virus-like particles. J Virol. 1997. 78, 1861-1866), the levels of expression are low, either secreted or intracellularly, hindering the purification process.

In parallel, several variants of the DENe protein have been obtained in bacteria. One of them was the C-terminal portion of the E protein of the JEV fused to a protein of the Tryptophan metabolism (TrpE) of *E. coli.* This protein was produced as inclusion bodies and was recognized by neutralizing monoclonal antibodies (Mabs) using

immunodetection techniques. However, pure preparations of this protein were unable to develop neutralizing antibodies and to protect against viral challenge (Mason P.W., Zogel M.V., Semproni A.R., et al. The antigenic structure of dengue type 1 virus envelope and NS1 protein expressed in *E. coli*. J Gen Virol. 1990. 71: 2107-2114). In addition, another construction was made (Srivastava A.K., Morita K., Matsuo S., et al. Japanese encephalitis virus fusion protein with protein A expressed in *E. coli* confers protection in mice. Microbiol Immunol. 1991. 35: 863-870), that contains the protein A of *Staphylococcus aurius* fused to the C-terminal fragment of the E protein, followed by the N-terminal segment of the nonstructural protein of the JEV, the NS1. In this case the fused protein was soluble, facilitating its purification by affinity chromatography. Upon immunizing mice with this pure protein high neutralizing antibodies titers were obtained, which also inhibited haemagglutination and protected against the viral challenge with the JEV. Similar results were obtained using the DENe region of the DEN-2 fused to the protein A of *S. aureus* (Srivastava A. K., Putnak R. J., Warren R. L., Hoke C. H. 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.1995. 13: 1251-1258); however, it is not possible to use these preparations in humans due to the presence of the protein A, which has shown a high affinity for the human immunoglobulin G (IgG).

Finally, it has been reported a fusion protein that contains the B domain of the DENe protein of DEN-2 and the maltose binding protein (MBP) of *E. coli* (Simmons M., Nelson W.M., Wu S.J., Hayes C.G. Evaluation of the protective efficacy of a recombinant dengue envelope B domain fusion protein against dengue 2 virus infection in mice. Am J Trop Med Hyg. 1998. 58: 655-662) denominated MBP-DomB. This protein variant was immunogenic in mice and elicited neutralizing antibodies.

In our case, the subject of this invention relies on the obtaining of chimeric sequences, as for instance, in the first case, the sequence coding for a region of the DENe protein linked to the N-terminal fragment of a mutated protein with dehydrogenase activity (MDH) from *Neisseria meningitidis*; in the second case, the sequence coding for a region of the DENe protein linked to the entire gene of the MDH protein in two different positions, and in the third case, the chimeric sequences are formed by two fragments of the DENe protein from two different viral serotypes fused to the same gene coding for the MDH protein. These chimeric chains when inserted into a suitable vector, give rise to insoluble chimeric proteins within the bacterium's cytoplasm. These proteins are then capable to elicit high levels of neutralizing antibodies against DEN, inhibitors of the viral hemagglutination and to protect immunized mice against viral challenge.

With regards to the insolubility of the aforementioned proteins, an easy scale-up folding process was achieved *in vitro*, as well as the expression and purification processes which

were superior to those used by Simmons et al, 1998. On the other hand, the serotype specificity of the antibodies is demonstrated, generated by immunization of mice with these proteins, at the level of neutralization, inhibition of hemaglutination and ELISA, using doses lower than those employed by Simmons et al, 1998. This fact constitutes the first report on the expression of insoluble DENe proteins in *E. coli* capable of stimulating a functionl immune response.

In addition, considering the results obtained with the dimeric variant, it is possible to generate serotype-specific antibodies with the same molecule for two different viral serotypes, capable of neutralizing viral infection and protect mice against viral challenge.

Concerning the MDH protein, a search for homology with other sequences was done in the EMBL data base, revealing that the first 110 amino acids are highly similar to the lipoic binding domain region and the flexible hinge of the dihydrolipoamide acetyltransferase (E2 enzyme of the pyruvate dehydrogenase complex and α-cetoglutarate dehydrogenase), and the rest of the protein is highly similar to the lipoamide dehydrogenase (LPDH), enzyme E3 of said complexes (Stephens, P. E; H. M. Darlinson,andJ. R. Guest.,1983. The Pyruvate dehydrogenase complex of *E. coli*. Eur. J. Biochem. 133: 155-162.

On the other hand, it was also found that patients with Primary Biliary Cirrhosis (PBC) produced anti-mitochondrial autoantibodies, specific for the lipoic acid binding site, common among these proteins (Gershwin ME, Mackay IR, Sturgess A, Coppel RL. Identification and specificity of a cDNA encoding the 70 KDa mitochondrial antigen recognized in primary biliary cirrhosis. J Immunol 1987;138:3525-31). Therefore, we decided to mutate this region within the protein to avoid any autoimmune response when immunized in humans as chimeric proteins. The mutated MDH protein of our invention was used in a Phase I clinical trial and showed to be safe and immunogenic in humans, and also was not recognized by sera of patients with PBC (Pérez, A., F. Dickinson, Z. Cinza, A. Ruíz, T. Serrano, J. Sosa, S. González, Y. Gutiérrez, C. Nazábal, O. Gutiérrez, D. Guzmán, M. Díaz, M. Delgado, E. Caballero, G. Sardiñas, A. Alvarez, A. Martín, G. Guillén, R. Silva. Safety and preliminary immunogenicity of the recombinant outer membrane protein of *Neisseria meningitidis* in human volunteers. Biotech. Appl. Biochem. 34: 121-125). However, the possible use of the MBP in humans has not been demonstrated yet (Simmons M., Nelson W.M., Wu S.J., Hayes C.G. Evaluation of the protective efficacy of a recombinant dengue envelope B domain fusion protein against dengue 2 virus infection in mice. Am J Trop Med Hyg. 1998. 58: 655-662).

Detailed description of the invention

In this invention is described the obtaining of chimeric nucleotide chains that when introduced into an expression vector, give rise to chimeric proteins capable of inducing a

serotype-specific humoral immune response and protecting against the infection by Dengue virus, as for instance, the sequence coding for a region of the DENe protein from each one of the viral serotypes of the Dengue virus, linked to the N-terminal fragment of a mutated protein with dehydrogenase activity (MDH) from *Neisseria meningitidis*; in the second case, the sequence coding for a region of the DENe protein linked to the entire gene of the MDH protein in two different positions: within one site of the sequence coding for the structural domain of the MDH protein (lipoic acid binding domain and the 3' end of the gen), and in the third case, the chimeric sequences are formed by two fragments of the DENe protein from two different viral serotypes, DEN-2 and DEN-4, in two different positions of the MDH gen: one within a particular site of the sequence coding for the lipoic acid binding domain (serotype 4) and the other in the 3' end of the MDH gen (serotype 2). This was called a dimeric construct.

This chimeric proteins were obtained insoluble within the bacterium's cytoplasm. A purification process by immobilazed metal affinity chromatography (IMAC) was done , which led to obtain pure proteins for immunogenicity studies.

When antigenicity results were analyzed a strong recognition of all the recombinant chimeric proteins for the hyperimmune ascitic liquids (HMAF) anti-DEN was demonstrated, being higher for the case of the fusion to the entire MDH gene, which evidences a positive effect on the folding of the region from the DENe protein given by the MDH. In the cases where the serotype 2 was used, all recombinant proteins obtained were recognized by a serotype-specific neutralizing antibody (3H5), being also higher for the case of the fusion to the entire MDH gene, as well as in the dimeric protein. It was also observed that the recognition for the HMAF from the homologous serotype in each case was significantly higher than the recognition for the HMAF from the heterologous serotypes, evidencing the exposure of serotype-specific epitopes and permitting thus its use as a diagnostic mean for Dengue and serotyping.

When all the recombinant chimeric proteins were immunized in mice a neutralizing and protective response was obtained. Highest neutralizing titers were obtained with the sequences fused to the entire gene of the MDH and with the dimeric protein, independently of the position of the fragment from the DENe protein. This showed an immunopotentiator effect of the immune response mediated by the MDH that can be explained by the influence in the folding of the DENe protein reflected in the antigenicity results obtained. It was also demonstrated for the first time, and contrary to the previous state of the art, that the insolubility of these proteins do not affect the capacity of generating a suitable immune response.

The immune response produced in all the cases was serotype-specific (antibodies against the immunized homologous serotype) in the viral neutralization, the hemoagglutination inhibition and ELISA. The generation of serotype-specific antibodies means that they are not capable to recognize antigenic determinants from virus of heterologous serotypes that favour the immunoenhacement phenomenum. This characteristic is of great importance for the development of a vaccine candidate against the Dengue virus since the recognition of antibodies to heterologous serotypes could be one of the causes for the Hemorrhagic Dengue Fever (HDF).

Besides it was showed the induction of antibodies against two viral serotypes after immunization with just one of the chimeric proteins, which permits the formulation of a vaccine candidate against the four serotypes, using only two of our available recombinant chimeric proteins.

The obtaining of the mutant MDH protein consisted of the elimination of the lipoic acid binding site in the sequence ETDKAT, based on the covalent binding of this fatty acid with the epsilon-amine groups of lysine (K) (Tuaillon N, Andre C, Briand JP et al. A lipoyl synthetic octadecapeptide of dihydrolipoamide acetyltransferase specifically recognized by anti-M2 autoantibodies in Primary Biliary Cirrhosis. J Immunol 1992; 148:445-50).

The mutagenesis was done by using PCR with a pair of primers to amplify the N-terminal region (from the start codon of the lpdA gene until the lipoic acid binding site, 135 bp) and the C-terminal of the protein (from the lipoic acid binding site until the 3' end of the gene); thus, being eliminated the possibility to generate autoimmune reactions, as demonstrated in the human clinical trials.

Deposit of the biological material

Plasmids PLL1, PLL2, PLL3, PLH1, PLH2, PLH3,PAZ1, PAZ2, PAZ3, PID1, PID2 and PID3 were deposited according to the Budapest Treaty in the Belgian Coordinated collection of Microorganism – BCCM™ , LMBP-COLLECTION, and with the date and under the access numbers (pending).

Description of Figures

Figure 1. Cloning strategy of the E2 fragment to obtain PLL1.

DENe2: Fragment of the envelope protein of DEN-2.

N-term: Nucleotide sequence that codifies for the first 45 amino acids of the MDH protein.

Figure 2. Cloning strategy of the E2 fragment to obtain PLL2.

DENe2: Fragment of the envelope protein of DEN-2.

MDH: dehydrogenase mutant.

Figure 3. Cloning strategy of the E2 fragment to obtain PLL3.

DENe2: Fragment of the envelope protein of DEN-2.

MDH: dehydrogenase mutant.

Figure 4. Cloning strategy of the E1 fragment to obtain PLH1.

DENe1: Fragment of the envelope protein of DEN-1.

N-term: Nucleotide sequence that codifies for the first 45 amino acids of the MDH protein.

Figure 5. Cloning strategy of the E1 fragment to obtain PLH2.

DENe1: Fragment of the envelope protein of DEN-1.

N-term: Nucleotide sequence that codifies for the first 45 amino acids of the MDH protein.

Figure 6. Cloning strategy of the E1 fragment to obtain PLH3.

DENe1: Fragment of the envelope protein of DEN-1.

MDH: dehydrogenase mutant.

Figure 7. Cloning strategy of the E3 fragment to obtain PAZ1.

DENe3: Fragment of the envelope protein of DEN-3.

N-term: Nucleotide sequence that codifies for the first 45 amino acids of the MDH protein.

Figure 8. Cloning strategy of the E3 fragment to obtain PAZ2.

DENe3: Fragment of the envelope protein of DEN-3.

MDH: dehydrogenase mutant.

Figure 9. Cloning strategy of the E3 fragment to obtain PAZ3.

DENe3: Fragment of the envelope protein of DEN-3.

MDH: dehydrogenase mutant.

Figure 10. Cloning strategy of the E4 fragment to obtain PID1.

DENe4: Fragment of the envelope protein of DEN-4.

N-term: Nucleotide sequence that codifies for the first 45 amino acids of the MDH protein.

Figure 11. Cloning strategy of the E4 fragment to obtain PID2.

DENe4: Fragment of the envelope protein of DEN-4.

MDH: dehydrogenase mutant.

Figure 12. Cloning strategy of the E4 fragment to obtain PID3.

DENe4: Fragment of the envelope protein of DEN-4.

MDH: dehydrogenase mutant.

Figura 13. Cloning strategy to obtain PD4D2.

DENe4: Fragment of the envelope protein of DEN-4.

DENe2: Fragment of the envelope protein of DEN-2.

MDH: dehydrogenase mutant.

EXAMPLES OF PERFORMANCE

EXAMPLE 1. Obtaining of PLL1

The nucleotide sequence that codifies for the amino acids from 286 to 426 of the envelope protein from the DEN-2 virus (Sec. Id. No. 22) was amplified with the oligonucleotides identified in the list of sequences as Sequence No.1 and Sequence No. 2 from the DEN-2 virus strain genotype Jamaica (Deubel V., Kinney R.M., Trent D.W. Nucleotide sequence and deduced amino acid sequence of the nonstructural proteins of Dengue type 2 virus, Jamaica genotype: Comparative analysis of the full-length genome.Virology 1988.165:234-244).

The vector was created by digestion of the pM108 His plasmid with *Xba I/Bam HI*, which contains the nucleotide sequence that codifies for the N-terminal region of the MDH and for a sequence of 6 histidines (Sequence No. 23). Upon ligation, the potential recombinants were analyzed by restriction enzyme digestion and positive clones were sequenced to check up the junctions. Competent cells W3110 (Hill C.W., Harnish B.W. 1982. Transposition of a chromosomal segment bounded by redundant rRNA genes in *Escherichia coli*. J Bacteriology. 149:449-457) were transformed with the selected clone, called pLL1 (Fig.1 and Sequence No. 24). Upon growing the colony in Luria Bertani (LB) medium, a SDS-PAGE of the cellular lysate was done. As a result a 25 kDA band was obtained, which accounted for 10% of the total cellular proteins. The size of the protein obtained corresponded to the sum of the N-terminal region from the MDH protein and the DENe protein fragment from the DEN-2 virus. The protein was recognized in Immunoblotting by polyclonal antibodies (PA) anti-DEN-2 contained in the HMAF. This protein was denominated PLL1 (Sequence No. 25).

EXAMPLE 2. Purification of the PLL1 protein

The biomass obtained from the *E. coli* strain transformed with pLL1 and grown at 37°C was disrupted by French press. The recombinant protein was obtained preponderantly as insoluble form associated to the pellet of the cellular disruption. From the pellet the protein was extracted with urea 6 M and the supernatant, containing the PLL1 protein, was loaded onto a G-25 column to eliminate the chaotropic agent. The fraction obtained was then loaded onto the Chelating-sepharose FF column (*Pharmacia, UK*), in the presence of Cu^{++} ions. The protein was eluted with Imidazole 50 mM and the obtained volume was loaded onto a G-25 column to finally obtain the protein in the formulation buffer NaCl 100 mM, KCl_2 2 mM, Na_2HPO_4 10 mM, pH 7.2, KH_2PO_4 1 mM (PBS). This preparation was used for immunological studies.

EXAMPLE 3. Antigenic characterization of PLL1

The purified fraction of PLL1 was characterized either by its recognition by different polyclonal sera and/or murine monoclonal antibodies, as well as by positive human sera to Dengue (table 1).

Table 1. Reactivity of PLL1 protein to monoclonal and polyclonal antibodies.

Abs**	*Specificity****	*PLL1**
HMAF	DEN-1	+
HMAF	DEN-2	++
HMAF	DEN-3	-
HMAF	DEN-4	-
HMAF	EEE	-
HMAF	YFV	-
HMAF	SLV	-
Mab 3H5	NT	+

* A total of 10 μg of purified PLL1 was applied. The intensity of the signal obtained was evaluated from + to ++.
** HMAF were used 1:100 while the Mab 3H5 was used in dilution 1:1000.
*** EEE: Equine Encephalitis virus. YFV: Yellow Fever virus. SLV: Saint Louis Encephalitis virus. NT: Neutralizing specific-serotype.

The highest recognitions in Dot blotting were obtained with the HMAF anti-DEN-2. This result coincides with the fact that the cloned region belongs to serotype 2. The recognition by HMAF against the other serotypes was less than in the case of serotype 2, in decreasing order: DEN-1, DEN-3 and DEN-4. The antibodies generated by other flavivirus like the Yellow Fever virus and the Saint Louis Encephalitis virus had not any recognition at all. On the other hand, the Mab 3H5 had reactivity indeed. This recognition by Western blotting relied on disulphide bond since when the sample was reduced the signal was lost. Finally, the reactivity against three human sera of high titers and three of low titers against DEN-2 was measured, achieving a substantial signal in both cases by Western blotting and Dot blotting.

EXAMPLE 4. Characterization of the antibody response generated by PLL1

A total of 25 Balb/c mice were i.p immunized with 35 ug of purified PLL1 in Freund adjuvant; 10 animals were bled after four doses and the antibodies anti-DEN were evaluated by ELISA. High antibody titers against DEN-2 were obtained while, no reactivity was obtained against the rest of the serotypes (table 2 and table 5). In addition, the hemagglutination inhibition assay (HIA) was done and only positive titers were found against DEN-2 (table 3 and table 5). Finally, the *in vitro* neutralization assay was done and neutralization titers of 1:320 against DEN-2 were obtained. However, no neutralization of the viral infection was found against the rest of the serotypes (table 4 and table 5). These results indicate the high serotype-specificity of the antibodies elicited by PLL1.

Table 2. Antibody titers against DEN-2 from the sera obtained upon immunization of mice with PLL1.

Mouse	Titers anti-DEN-2 PLL1	Titers anti-DEN-2 PBSControl (-)
1	1/ 128 000	< 1: 100
2	1/ 64 000	< 1: 100
3	1/ 64 000	< 1: 100
4	1/ 128 000	< 1: 100
5	1/ 32 000	< 1: 100
6	1/ 32 000	< 1: 100
7	1/ 64 000	< 1: 100
8	1/ 32 000	< 1: 100
9	1/ 128 000	< 1: 100
10	1/ 512 000	< 1: 100

Table 3. Titers by HI of the sera from the animals immunized with PLL1.

Mouse	Titers by HI* anti-DEN-2 PLL1	Titers by HI anti-DEN-2 PBS C(-)
1	< 1: 5	< 1: 5
2	>1: 640	< 1: 5
3	1: 320	< 1: 5
4	1: 320	< 1: 5
5	>1: 640	< 1: 5
6	>1: 640	< 1: 5
7	>1: 640	< 1: 5
8	1: 320	< 1: 5
9	>1: 640	< 1: 5
10	< 1: 5	< 1: 5

*The HI titers were defined as the highest dilution capable of inhibiting the hemagglutination of goose's erythrocytes against 8 hemagglutination viral units.

Table 4. Viral neutralization assay with the sera of animals immunized with PLL1.

Mouse	Neutralizing titers* anti-DEN-2 PLL1	Neutralizing titers anti-DEN-2 PBS C(-)
1	1: 320	< 1: 5
2	1: 320	< 1: 5
3	1: 320	< 1: 5
4	1: 320	< 1: 5
5	1: 80	< 1: 5
6	1: 160	< 1: 5
7	1: 320	< 1: 5
8	1: 40	< 1: 5
9	1: 160	< 1: 5
10	1: 320	< 1: 5

* The neutralizing titers were defined as the highest dilution of serum having 50% reduction of the number of viral plaques.

Table 5. Cross-reactivity assay against all the viral serotypes by ELISA, HI and viral neutralization with the sera of animals immunized with PLL1.

Mixture of sera *	ELISA (anti-DEN-1)	ELISA (anti DEN-2)	ELISA (anti-DEN-3)	ELISA (anti-DEN- 4)
1 (PLL1)	< 1/100	> 1: 128 000	< 1/100	< 1/100
2 (PLL1)	< 1/100	1: 128 000	< 1/100	< 1/100

Mixture of sera *	HI**anti-DEN-1	HI anti-DEN-2	HI anti-DEN-3	HI anti-DEN-4
PLL1	< 1/ 5	>1/ 320	< 1/5	< 1/5

Mixture of sera *	Neutralizing titers *** anti-DEN-1	Neutralizing titers anti-DEN-2	Neutralizing titers anti-DEN-3	Neutralizing titers anti-DEN-4
1 (PLL1)	< 1: 5	1: 320	< 1: 5	< 1: 5
2 (PLL1)	< 1: 5	1: 160	< 1: 5	< 1: 5

* Each mixture was formed by three sera.
** The HI titers were defined as the highest dilution capable of inhibiting the hemagglutination of goose's erythrocytes against 8 hemagglutination viral units.
*** The neutralizing titers were defined as the highest dilution of serum having 50% reduction of the number of viral plaques.

EXAMPLE 5. Obtaining of PLL2

The nucleotide sequence that codifies for the amino acids from 286 to 426 of the envelope protein from the DEN-2 virus (Sec. Id. No. 22) was amplified with the oligonucleotides identified in the list of sequences as Sequence No.1 and Sequence No.

3 from the DEN-2 virus strain genotype Jamaica (Deubel V., Kinney R.M., Trent D.W. Nucleotide sequence and deduced amino acid sequence of the nonstructural proteins of Dengue type 2 virus, Jamaica genotype: Comparative analysis of the full-length genome.Virology 1988.165:234-244).

The vector was created by digestion of the pM84 His plasmid with *Xba I/ Eco RI*, which contains the nucleotide sequence that codifies for the MDH protein and for a sequence of 6 histidines (Sequence No. 26). This digestion permits the insertion of the amplified fragment by PCR within the coding region for a structural domain of the MDH protein. Upon ligation, the potential recombinants were analyzed by restriction enzyme digestion and positive clones were sequenced to check up the junctions. Competent cells MM294 (Hanahan D. 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. 166:557-580) were transformed with the selected clone, called pLL2 (Fig. 2 and Sequence No. 27). Upon growing the colony in LB medium, a SDS-PAGE of the cellular lysate was done. As a result a 80 kDA band was obtained, which accounted for 10% of the total cellular proteins. The size of the protein obtained corresponded to the sum of the size of the MDH protein and the DENe protein fragment from the DEN-2 virus. The protein was recognized in Immunoblotting by a HMAF anti-DEN-2 and was denominated PLL2 (Sequence No. 28).

EXAMPLE 6. Purification of the PLL2 protein

The biomass obtained from the *E. coli* strain transformed with pLL2 and grown at 37^0C was disrupted by French press. The recombinant protein was obtained in both forms: soluble and insoluble. From the soluble fraction a metal ions-affinity chromatography was done using theChelating-sepharose FF column previously coupled with Cu^{++} ions. The column was washed using Imidazolee 15 mM and theprotein was eluted with Imidazolee 100 mM. On the other hand, the protein associated to the insoluble fraction was extracted using Urea 8 M, and the supernatant, containing the PLL2 protein, was loaded onto a G-25 column to eliminate the chaotropic agent . The fraction obtained was then loaded onto theChelating-sepharose FF column (*Pharmacia, UK*), in the presence of Cu^{++} ions. The protein was eluted with Imidazolee 100 mM. Finally, the pure fraction of each form of the protein were loaded onto a G-25 column to obtain the protein in the formulation buffer NaCl 100 mM, KCl_2 2 mM, Na_2HPO_4 10 mM, pH 7.2, KH_2PO_4 1 mM (PBS). This preparations were used for immunological studies.

EXAMPLE 7. Antigenic characterization of PLL2

The purified fraction of PLL2 was characterized either by its recognition by different polyclonal sera and/or murine monoclonal antibodies, as well as by positive human sera to Dengue (table 6).

The highest recognitions in Dot blotting were obtained with the HMAF anti-DEN-2. The recognition by HMAF against the other serotypes was less than in the case of serotype 2, in decreasing order: DEN-1, DEN-3 and DEN-4. The antibodies generated by other flavivirus like the Yellow Fever virus and the Saint Louis Encephalitis virus had not any recognition at all. Nevertheless, related to the Mab 3H5, a great reactivity was observed (even higher than those obtained with the PLL1) either by Dot blot and Western blot. Contrariously to the PLL1 results, the recognition with the Mab 3H5 was the same when the reducing agents are present in the sample, indicating a possible conformational difference between both proteins. Finally, the reactivity against three human sera of high titers and three of low titers against DEN-2 was measured, achieving a substantial signal in both cases by Western blotting and Dot blotting.

Table 6. Reactivity of PLL2 protein to monoclonal and polyclonal antibodies.

Abs**	*Specificity****	*PLL2 sol, ins**
HMAF	DEN-1	++
HMAF	DEN-2	+++
HMAF	DEN-3	-
HMAF	DEN-4	-
HMAF	EEE	-
HMAF	YFV	-
HMAF	SLV	-
Mab 3H5	NT	+++

*A total of 10 μg of purified PLL2 was applied. The intensity of the signal obtained was evaluated from + to ++.
** HMAF were used 1:100 while the Mab 3H5 was used in dilution 1:1000.
*** EEE: Equine Encephalitis virus. YFV: Yellow Fever virus. SLV: Saint Louis Encephalitis virus. NT: Neutralizing specific-serotype.

EXAMPLE 8. Characterization of the antibody response generated by PLL2

A total of 25 Balb/c mice were i.p immunized with 35 ug of purified PLL2 in Freund adjuvant; 10 animals were bled after four doses and the antibodies anti-DEN were evaluated by ELISA. High antibody titers against DEN-2 were obtained while, no reactivity was obtained against the rest of the serotypes (table 7 and table 10). In addition, the hemagglutination inhibition assay (HI) was done and only positive titers were found against DEN-2 (table 8 and table 10). Finally, the *in vitro* neutralization assay was done and neutralization titers of 1: 1280 against DEN-2 were obtained . Similarly to the results obtained with PLL1, no neutralization of the viral infection was found against the rest of the serotypes (table 9 and table 10). On the other hand, the results obtained with both variants of PLL2 were similar, indicating that the solubility status of the protein do not influece in the capacity of generating functional antibodies.

Table 7. Antibody titers against DEN-2 from the sera obtained upon immunization of mice with PLL2 soluble and insoluble.

Mouse	**Titers anti-DEN-2 (PLL2)**		**Titers anti-DEN-2 PBS C(-)**
	PLL2 s	**PLL2 ins**	
1	>1: 128 000	1: 64000	< 1: 100
2	1: 128 000	>1: 128 000	< 1: 100
3	>1: 128 000	1: 128 000	< 1: 100
4	>1: 128 000	>1: 128 000	< 1: 100
5	1:64 000	>1: 128 000	< 1: 100
6	>1: 128 000	1: 128 000	< 1: 100
7	1: 64000	>1: 128 000	< 1: 100
8	>1: 128 000	1: 64000	< 1: 100
9	>1: 128 000	1: 64000	< 1: 100
10	1: 128 000	>1: 128 000	< 1: 100

Table 8. Titers by HI of the sera from the animals immunized with PLL2 soluble and insoluble.

Mouse	Titers by HI* anti-DEN-2 (PLL2)		Titers by HI anti-DEN-2 PBS C(-)
	PLL2 s	PLL2 ins	
1	>1: 640	>1: 640	< 1: 5
2	>1: 640	>1: 640	< 1: 5
3	1: 320	>1: 640	< 1: 5
4	>1: 640	1: 320	< 1: 5
5	1: 320	< 1: 5	< 1: 5
6	>1: 640	>1: 640	< 1: 5
7	>1: 640	1: 320	< 1: 5
8	< 1: 5	1: 320	< 1: 5
9	1: 320	>1: 640	< 1: 5
10	>1: 640	>1: 640	< 1: 5

*The HI titers were defined as the highest dilution capable of inhibiting the hemagglutination of goose's erythrocytes against 8 hemagglutination viral units.

Table 9. Viral neutralization assay with the sera of animals immunized with PLL2 soluble and insoluble.

Mouse	Neutralizing titers* anti-DEN-2 (PLL2)		Neutralizing titers anti-DEN-2 PBS C(-)
	PLL2 s	PLL2 ins	
1	>1: 1280	>1: 1280	>1: 1280
2	>1: 1280	>1: 1280	< 1: 5
3	>1: 1280	1: 640	< 1: 5
4	1: 640	>1: 1280	< 1: 5
5	1: 640	1: 640	< 1: 5
6	>1: 1280	>1: 1280	< 1: 5
7	>1: 1280	>1:1280	< 1: 5
8	>1: 1280	>1: 1280	< 1: 5
9	>1: 1280	>1: 1280	< 1: 5
10	>1: 1280	>1: 1280	< 1: 5

* The neutralizing titers were defined as the highest dilution of serum having 50% reduction of the number of viral plaques.

Table 10. Cross-reactivity assay against all the viral serotypes by ELISA, HI and viral neutralization with the sera of animals immunized with PLL2 soluble and insoluble.

Mixture of sera *	ELISA (anti-DEN-1)	ELISA (anti DEN-2)	ELISA (anti-DEN-3)	ELISA (anti-DEN- 4)
1 (PLL2 sol.)	< 1/100	> 1: 128 000	< 1/100	< 1/100
2 (PLL2 sol.)	< 1/100	1: 64000	< 1/100	< 1/100
1 (PLL2 ins.)	< 1/100	1: 64000	< 1/100	< 1/100
2 (PLL2 ins.)	< 1/100	> 1: 128 000	< 1/100	< 1/100

Mixture of sera *	HI**anti-DEN-1	HI anti-DEN-2	HI anti-DEN-3	HI anti-DEN-4
PLL2 sol.	< 1/ 5	>1/ 320	< 1/5	< 1/5
PLL2 ins.	< 1/ 5	>1/ 320	< 1/5	< 1/5

Mixture of sera *	Neutralizing titers *** anti-DEN-1	Neutralizing titers anti-DEN-2	Neutralizing titers anti-DEN-3	Neutralizing titers anti-DEN-4
1 (PLL2)	< 1: 5	1: 320	< 1: 5	< 1: 5
2 (PLL2)	< 1: 5	1: 160	< 1: 5	< 1: 5

* Each mixture was formed by three sera.
** The HI titers were defined as the highest dilution capable of inhibiting the hemagglutination of goose's erythrocytes against 8 hemagglutination viral units.
*** The neutralizing titers were defined as the highest dilution of serum having 50% reduction of the number of viral plaques.

EXAMPLE 9. Obtaining of pLL3

The nucleotide sequence that codifies for the amino acids from 286 to 426 of the envelope protein from the DEN-2 virus (Sec. Id. No. 22) was amplified with the oligonucleotides identified in the list of sequences as Sequence No.4 and Sequence No.5 from the DEN-2 virus strain genotype Jamaica (Deubel V., Kinney R.M., Trent D.W. Nucleotide sequence and deduced amino acid sequence of the nonstructural proteins of Dengue type 2 virus, Jamaica genotype: Comparative analysis of the full-length genome.Virology 1988.165:234-244).

The vector was created by digestion of the pD4 plasmid with *Bam HI/Bam HI* which contains the nucleotide sequence that codifies for the MDH protein and for a sequence of 6 histidines without stop codon (Sequence No. 29). This digestion permits to the fusion of the amplified fragment by PCR after the C- terminal region for the MDH protein. Upon ligation, the potential recombinants were analyzed by restriction enzyme digestion and positive clones were sequenced to check up the junctions. Competent cells W3110 were transformed with the selected clone, called pLL3 (Fig. 3 and Sequence No. 30). Upon growing the colony in LB medium, a SDS-PAGE of the cellular lysate was done. As a result a 80 kDA band was obtained, which accounted for 20% of the total cellular proteins. The size of the protein obtained corresponded to the sum of the size of the MDH protein and the DENe protein fragment from the DEN-2 virus. The protein was recognized in Immunoblotting by a HMAFI anti-DEN-2 and was denominated PLL3 (Sequence No. 31).

EXAMPLE 10. Purification of the PLL3 protein

The biomass obtained from the *E. coli* strain transformed with pLL2 and grown at 37°C was disrupted by French press. The recombinant protein was obtained in both forms: soluble and insoluble. From the insoluble fraction, the protein was extracted using Urea 6 M, and the supernatant, containing the PLL3 protein, was loaded onto a G-25 column to eliminate the chaotropic agent. The fraction obtained was then loaded onto the Chelating-Sepharose FF column (*Pharmacia, UK*), in the presence of Cu^{++} ions. The colum was washed with Imidazolee 30 mM and the protein was eluted with Imidazole 100 mM. Finally, the pure fraction of the protein was loaded onto a G-25 column to obtain the protein in the formulation buffer (PBS). This preparation was used for immunological studies.

EXAMPLE 11. Antigenic characterization of PLL3

The purified fraction of PLL3 was characterized either by its recognition by different polyclonal sera and/or murine monoclonal antibodies, as well as by positive human sera to Dengue (table 11).

The highest recognitions in Dot blotting were obtained with the HMAF anti-DEN-2. The recognition by HMAF against the other serotypes was lower than in the case of serotype 2, in decreasing order: DEN-1, DEN-3 and DEN-4. The antibodies generated by other flavivirus like the Yellow Fever virus and the Saint Louis Encephalitis virus had not any recognition at all. Nevertheless, related to the Mab 3H5, a great reactivity was observed (similar to those obtained with the PLL2) either by Dot blot and Western blot. Contrariously to the PLL1 results, the recognition with the Mab 3H5 was the same when the reducing agents are present in the sample, indicating a possible conformational difference between both proteins. Finally, the reactivity against three human sera of high titers and three of low titers against DEN-2 was measured, achieving a substantial signal in both cases by Western blotting and Dot blotting. These results were similar to those obtained with PLL2.

Table 11. Reactivity of PLL3 protein to monoclonal and polyclonal antibodies.

Abs**	*Specificity****	*PLL3**
HMAF	DEN-1	++
HMAF	DEN-2	+++
HMAF	DEN-3	-
HMAF	DEN-4	-

HMAF	EEE	-
HMAF	YFV	-
HMAF	SLV	-
Mab 3H5	NT	+++

*A total of 10 μg of purified PLL3 was applied. The intensity of the signal obtained was evaluated from + to ++.
** HMAF were used 1:100 while the Mab 3H5 was used in dilution 1:1000.
*** EEE: Equine Encephalitis virus. YFV: Yellow Fever virus. SLV: Saint Louis Encephalitis virus. NT: Neutralizing specific-serotype.

EXAMPLE 12. Characterization of the antibody response generated by PLL3

A total of 25 Balb/c mice were i.p immunized with 35 ug of purified PLL3 in Freund adjuvant; 10 animals were bled after four doses and the antibodies anti-DEN were evaluated by ELISA. High antibody titers against DEN-2 were obtained while, no reactivity was obtained against the rest of the serotypes (table 12 and table 15). In addition, the hemagglutination inhibition (HI) assay was done and only positive titers were found against DEN-2 (table 13 and table 15). Finally, the *in vitro* neutralization assay was done and neutralization titers of 1: 1280 against DEN-2 were obtained (table 14). No neutralization of the viral infection was found against the rest of the serotypes (table 15). Using the three tests, high levels of serotype-specific antibodies were detected , similar of those obtained after the immunization with the PLL2 protein.

Table 12. Antibody titers against DEN-2 from the sera obtained upon immunization of mice with PLL3.

Mouse	Titers anti-DEN-2 (PLL3)	Titers anti-DEN-2 PBS C(-)
1	1: 128 000	< 1: 100
2	>1: 128 000	< 1: 100
3	>1: 128 000	< 1: 100
4	1:64 000	< 1: 100
5	>1: 128 000	< 1: 100
6	1: 64 000	< 1: 100
7	>1: 128 000	< 1: 100
8	>1: 128 000	< 1: 100
9	1: 128 000	< 1: 100
10	>1: 128 000	< 1: 100

Table 13. Titers by HI of the sera from the animals immunized with PLL3.

Mouse	Titers by HI* anti-DEN-2 (PLL3)	Titers by HI anti-DEN-2 PBS C(-)
1	>1: 640	< 1: 5
2	1: 320	< 1: 5
3	1: 320	< 1: 5
4	>1: 640	< 1: 5
5	1: 320	< 1: 5
6	>1: 640	< 1: 5
7	>1: 640	< 1: 5
8	>1: 640	< 1: 5
9	1: 320	< 1: 5
10	>1: 640	< 1: 5

*The HI titers were defined as the highest dilution capable of inhibiting the hemagglutination of goose's erythrocytes against 8 hemagglutination viral units.

Table 14. Viral neutralization assay with the sera of animals immunized with PLL3.

Mouse	Neutralizing titers anti- DEN-2 PLL3	Neutralizing titers anti- DEN-2 PBS C(-)
1	>1: 1280	< 1: 5
2	>1: 1280	< 1: 5
3	>1: 1280	< 1: 5
4	1: 640	< 1: 5
5	>1: 1280	< 1: 5
6	>1: 1280	< 1: 5
7	>1: 1280	< 1: 5
8	>1: 1280	< 1: 5
9	1: 640	< 1: 5
10	>1: 1280	< 1: 5

* The neutralizing titers were defined as the highest dilution of serum having 50% reduction of the number of viral plaques.

Table 15. Cross-reactivity assay against all the viral serotypes by ELISA, HI and viral neutralization with the sera of animals immunized with PLL3.

Mixture of sera *	ELISA (anti-DEN-1)	ELISA (anti DEN-2)	ELISA (anti-DEN-3)	ELISA (anti-DEN- 4)
1(PLL3)	< 1/100	> 1: 128 000	< 1/100	< 1/100
2(PLL3)	< 1/100	> 1: 128 000	< 1/100	< 1/100

Mixture of sera *	HI**anti-DEN-1	HI anti-DEN-2	HI anti-DEN-3	HI anti-DEN-4
PLL3	< 1/ 5	>1/ 320	< 1/ 5	< 1/ 5

Mixture of sera *	Neutralizing titers *** anti-DEN-1	Neutralizing titers *** anti-DEN-2	Neutralizing titers *** anti-DEN-3	Neutralizing titers *** anti-DEN-4
1(PLL3)	< 1: 5	>1: 1280	< 1: 5	< 1: 5
2(PLL3)	< 1: 5	>1: 1280	< 1: 5	< 1: 5

* Each mixture was formed by three sera.
** The HI titers were defined as the highest dilution capable of inhibiting the hemagglutination of goose's erythrocytes against 8 hemagglutination viral units.
*** The neutralizing titers were defined as the highest dilution of serum having 50% reduction of the number of viral plaques.

EXAMPLE 13. Obtaining of pLH1

The nucleotide sequence that codifies for the amino acids from 286 to 426 of the envelope protein from the DEN-1 virus (Sec. Id. No. 32) was amplified with the oligonucleotides identified in the list of sequences as Sequence No.6 and Sequence No.7 from the DEN-1 virus strain genotype (Chu M.C., O'Rourke E.J., Trent D.W.Genetic relatedness among structural protein genes of dengue 1 virus strains.J. Gen. Virol.1989. 70:1701-1712).

The vector was created by digestion of the pM108 His plasmid with *Xba I/Bam HI*, which contains the nucleotide sequence that codifies for the N-terminal region of the MDH and for a sequence of 6 histidines (Sequence No. 23). Upon ligation, the potential recombinants were analyzed by restriction enzyme digestion and positive clones were sequenced to check up the junctions. Competent cells W3110 were transformed with the selected clone, called pLH1 (Fig. 4 and Sequence No.33). Upon growing the colony in Luria Bertani (LB) medium, a SDS-PAGE of the cellular lysate was done. As a result a 25 kDA band was obtained, which accounted for 10% of the total cellular proteins. The size of the protein obtained corresponded to the sum of the N-terminal region from the MDH protein and the DENe protein fragment from the DEN-1 virus. The protein was recognized in Immunoblotting by polyclonal antibodies (PA) anti-DEN-1 contained in the HMAF. This protein was denominated PLH1 (Sequence No.34).

EXAMPLE 14. Purification of the protein PLH1

The biomass obtained from the *E. coli* strain transformed with pLH1 and grown at 37°C was disrupted by French press. The recombinant protein was obtained preponderantly as insoluble form associated to the pellet of the cellular disruption. From the pellet, the

protein was extracted with urea 7 M and the supernatant, containing the PLH1 protein, was loaded onto a G-25 column to eliminate the chaotropic agent. The fraction obtained was then loaded onto the Chelating-sepharose FF column (*Pharmacia, UK*), in the presence of Cu^{++} ions. The protein was eluted with Imidazolee 60 mM and the obtained volume was loaded onto a G-25 column to finally obtain the protein in the formulation buffer (PBS). This preparation was used for immunological studies.

EXAMPLE 15. Antigenic characterization of PLH1

The purified fraction of PLH1 was characterized either by its recognition by different polyclonal sera and/or murine monoclonal antibodies, as well as by positive human sera to Dengue (table 16).

The highest recognitions in Dot blotting were obtained with the HMAF anti-DEN-1. The recognition by HMAF against the other serotypes was lower than in the case of serotype 1. The antibodies generated by other flavivirus like the Yellow Fever virus and the Saint Louis Encephalitis virus had not any recognition at all. Finally, the reactivity against three human sera of high titers and three of low titers against DEN-1 was measured, achieving a substantial signal in both cases by Western blotting and Dot blotting.

Table 16. Reactivity of PLH1 protein to monoclonal and polyclonal antibodies

Abs**	*Specificity****	*PLH1*
HMAF	DEN-1	++
HMAF	DEN-2	+
HMAF	DEN-3	-
HMAF	DEN-4	-
HMAF	EEE	-
HMAF	YFV	-
HMAF	SLV	-
Mab 3H5	NT	-

*A total of 10 μg of purified PLH1 was applied. The intensity of the signal obtained was evaluated from + to ++.
** HMAF were used 1:100 while the Mab 3H5 was used in dilution 1:1000.
*** EEE: Equine Encephalitis virus. YFV: Yellow Fever virus. SLV: Saint Louis Encephalitis virus. NT: Neutralizing specific-serotype.

EXAMPLE 16. Characterization of the antibody response generated by PLH1

A total of 25 Balb/c mice were i.p immunized with 35 ug of purified PLH1 in Freund adjuvant; 10 animals were bled after four doses and the antibodies anti-DEN were evaluated by ELISA. High antibody titers against DEN-1 were obtained while, no reactivity was obtained against the rest of the serotypes (table 17 and table 20). In addition, the HI assay was done and only positive titers were found against DEN-1 (table 18 and table 20). Finally, the *in vitro* neutralization assay was done and neutralization titers of 1:320 against DEN-1 were obtained. However, no neutralization of the viral infection was found against the rest of the serotypes (table 19 and table 20). These results indicate the high serotype-specificity of the antibodies elicited by PLH1.

Table 17. Antibody titers against DEN-1 from the sera obtained upon immunization of mice with PLH1.

Mouse	Titers anti-DEN-1 (PLH1)	Titers anti-DEN-1 PBS C(-)
1	1/ 64 000	< 1: 100
2	1/ 128 000	< 1: 100
3	1/ 64 000	< 1: 100
4	1/ 32 000	< 1: 100
5	1/ 32 000	< 1: 100
6	1/ 64 000	< 1: 100
7	1/ 128 000	< 1: 100
8	1/ 64 000	< 1: 100
9	1/ 128 000	< 1: 100
10	1/ 128 000	< 1: 100

Table 18. Titers by HI of the sera from the animals immunized with PLH1.

Mouse	Titers by HI* anti-DEN-1 (PLH1)	Titers by HI* anti-DEN-1 PBS C(-)
1	>1: 640	< 1: 5
2	1: 320	< 1: 5
3	>1: 640	< 1: 5
4	>1: 640	< 1: 5
5	>1: 640	< 1: 5
6	1:320	< 1: 5
7	1: 40	< 1: 5
8	1:320	< 1: 5
9	>1: 640	< 1: 5
10	< 1: 5	< 1: 5

*The HI titers were defined as the highest dilution capable of inhibiting the hemagglutination of goose's erythrocytes against 8 hemagglutination viral units.

Table 19. Viral neutralization assay with the sera of animals immunized with PLH1.

Mouse	Neutralizing titers* anti-DEN-1 (PLH1)	Neutralizing titers* anti-DEN-1 PBS C(-)
1	1: 80	< 1: 5
2	1: 320	< 1: 5
3	1: 40	< 1: 5
4	1: 320	< 1: 5
5	1: 80	< 1: 5
6	1: 160	< 1: 5
7	1: 320	< 1: 5
8	1: 320	< 1: 5
9	1: 320	< 1: 5
10	1: 320	< 1: 5

* The neutralizing titers were defined as the highest dilution of serum having 50% reduction of the number of viral plaques.

Table 20. Cross-reactivity assay against all the viral serotypes by ELISA, HI and viral neutralization with the sera of animals immunized with PLH1.

Mixture of sera *	ELISA (anti-DEN-1)	ELISA (anti DEN-2)	ELISA (anti-DEN-3)	ELISA (anti-DEN- 4)
1 (PLH1)	1: 128 000	< 1/100	< 1/100	< 1/100
2 (PLH1)	> 1: 128 000	< 1/100	< 1/100	< 1/100

Mixture of sera *	HI**anti-DEN-1	HI anti-DEN-2	HI anti-DEN-3	HI anti-DEN-4
PLH1	>1: 320	< 1/ 5	< 1/ 5	< 1/ 5

Mixture of sera *	Neutralizing titers *** anti-DEN-1	Neutralizing titers *** anti-DEN-2	Neutralizing titers *** anti-DEN-3	Neutralizing titers *** anti-DEN-4
1(PLH1)	1: 160	< 1: 5	< 1: 5	< 1: 5
2(PLH1)	1: 320	< 1: 5	< 1: 5	< 1: 5

* Each mixture was formed by three sera.
** The HI titers were defined as the highest dilution capable of inhibiting the hemagglutination of goose's erythrocytes against 8 hemagglutination viral units.
*** The neutralizing titers were defined as the highest dilution of serum having 50% reduction of the number of viral plaques.

EXAMPLE 17. Obtaining of pLH2

The nucleotide sequence that codifies for the amino acids from 286 to 426 of the envelope protein from the DEN-1 virus (Sequence No. 32) was amplified with the oligonucleotides identified in the list of sequences as Sequence No.6 and Sequence No.8

from a DEN-1 viral strain (Chu M.C., O'Rourke E.J., Trent D.W.Genetic relatedness among structural protein genes of dengue 1 virus strains. J. Gen. Virol.1989. 70:1701-1712).

The vector was created by digestion of the pM84 His plasmid with *Xba I/ Eco RI*, which contains the nucleotide sequence that codifies for the MDH protein and for a sequence of 6 histidines (Sequence No. 26). This digestion permits the insertion of the amplified fragment by PCR within the coding region for a structural domain of the MDH protein. Upon ligation, the potential recombinants were analyzed by restriction enzyme digestion and positive clones were sequenced to check up the junctions. Competent cells MM294 were transformed with the selected clone, called pLH2 (Fig. 5 and Sequence No.35). Upon growing the colony in LB medium, a SDS-PAGE of the cellular lysate was done. As a result a 80 kDA band was obtained, which accounted for 20% of the total cellular proteins. The size of the protein obtained corresponded to the sum of the size of the MDH protein and the DENe protein fragment from the DEN-1 virus. The protein was recognized in Immunoblotting by a HMAF anti-DEN-1 and was denominated PLH2 (Sequence No.36).

EXAMPLE 18. Purification of the PLH2 protein

The biomass obtained from the *E. coli* strain transformed with pLH2 and grown at 37°C was disrupted by French press. The recombinant protein was obtained in both forms: soluble and insoluble. The protein associated to the insoluble fraction was extracted using Urea 7 M, and the supernatant, containing the PLH2 protein, was loaded onto a G-25 column to eliminate the chaotropic agent . The fraction obtained was then loaded onto theChelating-sepharose FF column (*Pharmacia, UK*), in the presence of Cu^{++} ions. The column was washed with Imidazolee 40 mM and the protein was eluted with Imidazole 100 mM. Finally, the pure fraction was loaded onto a G-25 column to obtain the protein in the formulation (PBS). This preparation was used for immunological studies.

EXAMPLE 19. Antigenic characterization of PLH2

The purified fraction of PLH2 was characterized either by its recognition by different polyclonal sera and/or murine monoclonal antibodies, as well as by positive human sera to Dengue (table 21).

The highest recognitions in Dot blotting were obtained with the HMAF anti-DEN-1 (higher than those obtained with PLH1). The recognition by HMAF against the other serotypes was lower than in the case of serotype 1. The antibodies generated by other flavivirus like the Yellow Fever virus and the Saint Louis Encephalitis virus had not any recognition at all. Finally, the reactivity against five human sera of high titers and three of low titers

against DEN-1 was measured, achieving a substantial signal in both cases by Western blotting and Dot blotting.

Table 21. Reactivity of PLH2 protein to monoclonal and polyclonal antibodies.

Abs**	*Specificity****	*PLH2*
HMAF	DEN-1	+++
HMAF	DEN-2	+
HMAF	DEN-3	-
HMAF	DEN-4	-
HMAF	EEE	-
HMAF	YFV	-
HMAF	SLV	-
Mab 3H5	NT	-

*A total of 10 μg of purified PLH2 was applied. The intensity of the signal obtained was evaluated from + to ++.
** HMAF were used 1:100 while the Mab 3H5 was used in dilution 1:1000.
*** EEE: Equine Encephalitis virus. YFV: Yellow Fever virus. SLV: Saint Louis Encephalitis virus. NT: Neutralizing specific-serotype.

EXAMPLE 20. Characterization of the antibody response generated by PLH2

A total of 25 Balb/c mice were i.p immunized with 20 ug of purified PLH2 in Freund adjuvant; 10 animals were bled after four doses and the antibodies anti-DEN were evaluated by ELISA. High antibody titers against DEN-1 were obtained while, no reactivity was obtained against the rest of the serotypes (table 22 and table 25). In addition, the HI assay was done and only positive titers were found against DEN-1 (table 23 and table 25). Finally, the *in vitro* neutralization assay was done and neutralization titers of 1: 1280 against DEN-1 were obtained (table 24). No neutralization of the viral infection was found against the rest of the serotypes. (table 25).

Table 22. Antibody titers against DEN-1 from the sera obtained upon immunization of mice with PLH2.

Mouse	**Titers anti-DEN-1 (PLH2)**	**Titers anti-DEN-1 PBS C(-)**
1	1: 128 000	< 1: 100

2	>1: 128 000	< 1: 100
3	1: 64000	< 1: 100
4	>1: 128 000	< 1: 100
5	1: 128 000	< 1: 100
6	1: 64000	< 1: 100
7	>1: 128 000	< 1: 100
8	1: 128 000	< 1: 100
9	>1: 128 000	< 1: 100
10	>1: 128 000	< 1: 100

Table 23. Titers by HI of the sera from the animals immunized with PLH2.

Mouse	Titers by HI* anti-DEN-1 (PLH2)	Titers by HI anti-DEN -1 PBS C(-)
1	< 1: 5	< 1: 5
2	>1: 640	< 1: 5
3	1: 320	< 1: 5
4	1: 320	< 1: 5
5	>1: 640	< 1: 5
6	>1: 640	< 1: 5
7	>1: 640	< 1: 5
8	1: 320	< 1: 5
9	>1: 640	< 1: 5
10	< 1: 5	< 1: 5

*The HI titers were defined as the highest dilution capable of inhibiting the hemagglutination of goose's erythrocytes against 8 hemagglutination viral units.

Table 24 . Viral neutralization assay with the sera of animals immunized with PLH2.

Mouse	Neutralizing titers* anti-DEN-1 (PLH2)	Neutralizing titers* anti-DEN-1. C(-)
1	>1: 1280	< 1: 5
2	>1: 1280	< 1: 5
3	>1: 1280	< 1: 5
4	>1: 1280	< 1: 5
5	>1: 1280	< 1: 5
6	>1: 1280	< 1: 5
7	>1: 1280	< 1: 5
8	>1: 1280	< 1: 5
9	1: 640	< 1: 5
10	>1: 1280	< 1: 5

* The neutralizing titers were defined as the highest dilution of serum having 50% reduction of the number of viral plaques.

Table 25. Cross-reactivity assay against all the viral serotypes by ELISA, HI and viral neutralization with the sera of animals immunized with PLH2.

Mixture of sera *	ELISA (anti-DEN-1)	ELISA (anti DEN-2)	ELISA(anti-DEN-3)	ELISA(anti-DEN- 4)
1 (PLH2)	1: 64 000	< 1/100	< 1/100	< 1/100
2 (PLH2)	> 1: 128 000	< 1/100	< 1/100	< 1/100

Mixture of sera *	HI**anti-DEN-1	HI anti-DEN-2	HI anti-DEN-3	HI anti-DEN-4
PLH2	>1/ 320	< 1/ 5	< 1/ 5	< 1/ 5

Mixture of sera *	Neutralizing titers*** anti-DEN-1	Neutralizing titers anti-DEN-2	Neutralizing titers anti-DEN-3	Neutralizing titers anti-DEN-4
1(PLH2)	>1: 1280	< 1: 5	< 1: 5	< 1: 5
2(PLH2)	>1: 1280	< 1: 5	< 1: 5	< 1: 5

* Each mixture was formed by three sera.
** The HI titers were defined as the highest dilution capable of inhibiting the hemagglutination of goose's erythrocytes against 8 hemagglutination viral units.
*** The neutralizing titers were defined as the highest dilution of serum having 50% reduction of the number of viral plaques.

EXAMPLE 21. Obtaining of pLH3

The nucleotide sequence that codifies for the amino acids from 286 to 426 of the envelope protein from the DEN-1 virus (Sec. Id. No. 32) was amplified with the oligonucleotides identified in the list of sequences as Sequence No.9 and Sequence No.10 from the DEN-1 virus strain (Chu M.C., O'Rourke E.J., Trent D.W.Genetic relatedness among structural protein genes of dengue 1 virus strain. J. Gen. Virol.1989. 70:1701-1712).

The vector was created by digestion of the pD4 plasmid with *Bam HI/Bam HI* which contains the nucleotide sequence that codifies for the MDH protein and for a sequence of 6 histidines without stop codon (Sequence No.29). This digestion permits the fusion of the amplified fragment by PCR after the C- terminal region for the MDH protein. Upon ligation, the potential recombinants were analyzed by restriction enzyme digestion and positive clones were sequenced to check up the junctions. Competent cells W3110 were transformed with the selected clone, called pLH3 (Fig. 6 and Sequence No.37). Upon growing the colony in LB medium, a SDS-PAGE of the cellular lysate was done. As a result a 80 kDA band was obtained, which accounted for 20% of the total cellular proteins. The size of the protein obtained corresponded to the sum of the size of the MDH protein and the DENe protein fragment from the DEN-1 virus. The protein was recognized

in Immunoblotting by a HMAF anti-DEN-1 and was denominated PLH3 (Sequence No.38).

EXAMPLE 22. Purification of the PLH3 protein

The biomass obtained from the *E. coli* strain transformed with pLH3 and grown at 37°C was disrupted by French press. The recombinant protein was obtained in both forms: soluble and insoluble. From the insoluble fraction, the protein was extracted using Urea 6 M, and the supernatant, containing the PLH3 protein, was loaded onto a G-25 column to eliminate the chaotropic agent. The fraction obtained was then loaded onto the Chelating-Sepharose FF column (*Pharmacia, UK*), in the presence of Cu^{++} ions. The colum was washed with Imidazolee 30 mM and the protein was eluted with Imidazole 250 mM. Finally, the pure fraction of the protein was loaded onto a G-25 column to obtain the protein in the formulation buffer (PBS). This preparation was used for immunological studies.

EXAMPLE 23. Antigenic characterization of PLH3

The purified fraction of PLH3 was characterized either by its recognition by different polyclonal sera and/or murine monoclonal antibodies, as well as by positive human sera to Dengue (table 26).

The highest recognitions in Dot blotting were obtained with the HMAF anti-DEN-1. The recognition by HMAF against the other serotypes was lower than in the case of serotype 1. The antibodies generated by other flavivirus like the Yellow Fever virus and the Saint Louis Encephalitis virus had not any recognition at all. Finally, the reactivity against three human sera of high titers and three of low titers against DEN-1 was measured, achieving a substantial signal in both cases by Dot blotting and Western blotting.

Table 26. Reactivity of PLH3 protein to monoclonal and polyclonal antibodies.

Abs**	*Specificity***	*PLH3*
HMAF	DEN-1	+++
HMAF	DEN-2	+
HMAF	DEN-3	-
HMAF	DEN-4	-

HMAF	EEE	-
HMAF	YFV	-
HMAF	SLV	-
Mab 3H5	NT	-

* A total of 10 μg of purified PLH3 was applied. The intensity of the signal obtained was evaluated from + to ++.
** HMAF were used 1:100 while the Mab 3H5 was used in dilution 1:1000.
*** EEE: Equine Encephalitis virus. YFV: Yellow Fever virus. SLV: Saint Louis Encephalitis virus. NT: Neutralizing specific-serotype.

EXAMPLE 24. Characterization of the antibody response generated by PLH3

A total of 25 Balb/c mice were i.p immunized with 20 ug of purified PLH3 in Freund adjuvant; 10 animals were bled after four doses and the antibodies anti-DEN were evaluated by ELISA. High antibody titers against DEN-1 were obtained while, no reactivity was obtained against the rest of the serotypes (table 27 and table 30). In addition, the HI assay was done and only positive titers were found against DEN-1 (table 28andtable 30). Finally, the *in vitro* neutralization assay was done and neutralization titers of 1: 1280 against DEN-1 were obtained (table 29). No neutralization of the viral infection was found against the rest of the serotypes (table 30).

Table 27. Antibody titers against DEN-1 from the sera obtained upon immunization of mice with PLH3.

Mouse	Titers anti-DEN-1 PLH3	Titers anti-DEN-1 PBSControl (-)
1	1: 64 000	< 1: 100
2	>1: 128 000	< 1: 100
3	1: 64 000	< 1: 100
4	>1: 128 000	< 1: 100
5	1: 128 000	< 1: 100
6	1: 128 000	< 1: 100
7	1: 128 000	< 1: 100
8	>1: 128 000	< 1: 100
9	1: 64 000	< 1: 100
10	>1: 128 000	< 1: 100

Table 28 . Titers by HI of the sera from the animals immunized with PLH3.

Mouse	Titers by HI* anti-DEN-1 PLH3	Titers by HI anti-DEN-1 PBS C(-)
1	1: 320	< 1: 5

2	>1: 640	< 1: 5
3	>1: 640	< 1: 5
4	1: 320	< 1: 5
5	1: 320	< 1: 5
6	>1: 640	< 1: 5
7	1: 320	< 1: 5
8	< 1: 5	< 1: 5
9	>1: 640	< 1: 5
10	>1: 640	< 1: 5

*The HI titers were defined as the highest dilution capable of inhibiting the hemagglutination of goose's erythrocytes against 8 hemagglutination viral units.

Table 29. Viral neutralization assay with the sera of animals immunized with PLH3.

Mouse	Neutralizing titers* anti- DEN-1 PLH3	Neutralizing titers* anti- DEN-1 PBS C(-)
1	>1: 1280	< 1: 5
2	>1: 1280	< 1: 5
3	>1: 1280	< 1: 5
4	1: 640	< 1: 5
5	1: 640	< 1: 5
6	>1: 1280	< 1: 5
7	>1: 1280	< 1: 5
8	>1: 1280	< 1: 5
9	>1: 1280	< 1: 5
10	>1: 1280	< 1: 5

*The neutralizing titers were defined as the highest dilution of serum having 50% reduction of the number of viral plaques.

Table 30. Cross-reactivity assay against all the viral serotypes by ELISA, HI and viral neutralization with the sera of animals immunized with PLH3.

Mixture of sera *	ELISA (anti-DEN-1)	ELISA (anti DEN-2)	ELISA (anti-DEN-3)	ELISA (anti-DEN- 4)
1(PLH3)	1: 128 000	< 1/100	< 1/100	< 1/100
2(PLH3)	> 1: 128 000	< 1/100	< 1/100	< 1/100

Mixture of sera *	HI**anti-DEN-1	HI anti-DEN-2	HI anti-DEN-3	HI anti-DEN-4
PLH3	>1/ 320	< 1/ 5	< 1/ 5	< 1/ 5

Mixture of sera *	Neutralizing titers*** anti-DEN-1	Neutralizing titers anti-DEN-2	Neutralizing titers anti-DEN-3	Neutralizing titers anti-DEN-4
1(PLH3)	>1: 1280	< 1: 5	< 1: 5	< 1: 5
2(PLH3)	>1: 1280	< 1: 5	< 1: 5	< 1: 5

* Each mixture was formed by three sera.
** The HI titers were defined as the highest dilution capable of inhibiting the hemagglutination of goose's erythrocytes against 8 hemagglutination viral units.
*** The neutralizing titers were defined as the highest dilution of serum having 50% reduction of the number of viral plaques.

EXAMPLE 25. Obtaining of pAZ1

The nucleotide sequence that codifies for the amino acids from 286 to 426 of the envelope protein from the DEN-3 virus (Seq. 39) was amplified with the oligonucleotides identified in the list of sequences as Sequence No. 11 and Sequence No.12 from the DEN-3 virus strain genotype (Osatomi K., Sumiyoshi H. Complete nucleotide sequence of dengue type 3 virus genome RNA. Virology.1990. 176(2):643-647).

The vector was created by digestion of the pM108 His plasmid with *Xba I/Bam HI*, which contains the nucleotide sequence that codifies for the N-terminal region of the MDH and for a sequence of 6 histidines (Sequence No. 23). Upon ligation, the potential recombinants were analyzed by restriction enzyme digestion and positive clones were sequenced to check up the junctions. Competent cells W3110 were transformed with the selected clone, called pAZ1 (Fig. 7 and Sequence No.40). Upon growing the colony in Luria Bertani (LB) medium, a SDS-PAGE of the cellular lysate was done. As a result a 25 kDA band was obtained, which accounted for 10% of the total cellular proteins. The size of the protein obtained corresponded to the sum of the N-terminal region from the MDH protein and the DENe protein fragment from the DEN-3 virus. The protein was recognized in Immunoblotting by polyclonal antibodies (PA) anti-DEN-3 contained in the HMAF. This protein was denominated PAZ1 (Sequence No.41).

EXAMPLE 26. Purification of the protein PAZ1

The biomass obtained from the *E. coli* strain transformed with pAZ1 and grown at 37°C was disrupted by French press. The recombinant protein was obtained preponderantly as insoluble form associated to the pellet of the cellular disruption. From the pellet, the protein was extracted with urea 7 M and the supernatant, containing the PLH1 protein, was loaded onto a G-25 column to eliminate the chaotropic agent. The fraction obtained was then loaded onto the Chelating-sepharose FF column (*Pharmacia, UK*), in the presence of Cu^{++} ions. The protein was eluted with Imidazolee 60 mM and the obtained

volume was loaded onto a G-25 column to finally obtain the protein in the formulation buffer (PBS). This preparation was used for immunological studies.

EXAMPLE 27. Antigenic characterization of PAZ1

The purified fraction of PAZ1 was characterized either by its recognition by different polyclonal sera and/or murine monoclonal antibodies, as well as by positive human sera to Dengue (table 31).

The highest recognitions in Dot blotting were obtained with the HMAF anti-DEN-3. The recognition by HMAF against the other serotypes was lower than in the case of serotype 3. The antibodies generated by other flavivirus like the Yellow Fever virus and the Saint Louis Encephalitis virus had not any recognition at all. Finally, the reactivity against three human sera of high titers and three of low titers against DEN-3 was measured, achieving a substantial signal in both cases by Dot blotting Western blotting.

Table 31. Reactivity of PAZ1 protein to monoclonal and polyclonal antibodies.

Abs**	*Specificity****	*PAZ1*
HMAF	DEN-1	-
HMAF	DEN-2	+
HMAF	DEN-3	++
HMAF	DEN-4	+
HMAF	EEE	-
HMAF	YFV	-
HMAF	SLV	-
Mab 3H5	NT	-

* A total of 10 μg of purified PAZ1 was applied. The intensity of the signal obtained was evaluated from + to ++.
** HMAF were used 1:100 while the Mab 3H5 was used in dilution 1:1000.
*** EEE: Equine Encephalitis virus. YFV: Yellow Fever virus. SLV: Saint Louis Encephalitis virus. NT: Neutralizing specific-serotype.

EXAMPLE 28. Characterization of the antibody response generated by PAZ1

A total of 25 Balb/c mice were i.p immunized with 35 ug of purified PAZ1 in Freund adjuvant; 10 animals were bled after four doses and the antibodies anti-DEN were evaluated by ELISA. High antibody titers against DEN-1 were obtained while, no reactivity

was obtained against the rest of the serotypes (table 32 and table 35). In addition, the HI assay was done and only positive titers were found against DEN-3 (table 33 and table 35). Finally, the *in vitro* neutralization assay was done and neutralization titers of 1:320 against DEN- 3 were obtained. However, no neutralization of the viral infection was found against the rest of the serotypes (table 34 and table 35). These results indicate the high serotype-specificity of the antibodies elicited by PAZ1.

Table 32. Antibody titers against DEN-3 from the sera obtained upon immunization of mice with PAZ1.

Mouse	Titers anti-DEN-3 PAZ1	Titers anti-DEN-3 PBSControl (-)
1	1/ 64 000	< 1: 100
2	1/ 128 000	< 1: 100
3	1/ 32 000	< 1: 100
4	1/ 64 000	< 1: 100
5	1/ 64 000	< 1: 100
6	1/ 128 000	< 1: 100
7	1/ 64 000	< 1: 100
8	1/ 64 000	< 1: 100
9	1/ 128 000	< 1: 100
10	1/ 128 000	< 1: 100

Table 33. Titers by HI of the sera from the animals immunized with PAZ1.

Mouse	Titers by HI* anti-DEN-3 PAZ1	Titers by HI anti-DEN-3 PBS C(-)
1	>1: 640	< 1: 5
2	1: 320	< 1: 5
3	1: 320	< 1: 5
4	1: 640	< 1: 5
5	< 1/ 5	< 1: 5
6	1: 320	< 1: 5
7	< 1/ 5	< 1: 5
8	1: 320	< 1: 5
9	>1: 640	< 1: 5
10	>1: 640	< 1: 5

*The HI titers were defined as the highest dilution capable of inhibiting the hemagglutination of goose's erythrocytes against 8 hemagglutination viral units.

Table 34. Viral neutralization assay with the sera of animals immunized with PAZ1.

Mouse	Neutralizing titers anti- DEN-3 PAZ1	Neutralizing titers anti-DEN-3 PBS C(-)
1	1: 160	< 1: 5

2	1: 320	< 1: 5
3	1: 320	< 1: 5
4	1: 320	< 1: 5
5	1: 40	< 1: 5
6	1: 40	< 1: 5
7	1: 320	< 1: 5
8	1: 320	< 1: 5
9	1: 160	< 1: 5
10	1: 320	< 1: 5

* The neutralizing titers were defined as the highest dilution of serum having 50% reduction of the number of viral plaques.

Table 35. Cross-reactivity assay against all the viral serotypes by ELISA, HI and viral neutralization with the sera of animals immunized with PAZ1.

Mixture of sera *	ELISA (anti-DEN-1)	ELISA (anti-DEN-2)	ELISA (anti-DEN-3)	ELISA (anti-DEN- 4)
1 (PAZ1)	< 1/100	< 1/100	1: 64 000	< 1/100
2 (PAZ1)	< 1/100	< 1/100	> 1: 128 000	< 1/100

Mixture of sera *	HI**anti-DEN-1	HI anti-DEN-2	HI anti-DEN-3	HI anti-DEN-4
PAZ1	< 1/ 5	< 1/ 5	>1: 320	< 1/ 5

Mixture of sera *	Neutralizing titers*** anti-DEN-1	Neutralizing titers anti-DEN-2	Neutralizing titers anti-DEN-3	Neutralizing titers anti-DEN-4
1(PAZ1)	< 1: 5	< 1: 5	1: 320	< 1: 5
2(PAZ1)	< 1: 5	< 1: 5	1: 320	< 1: 5

* Each mixture was formed by three sera.
** The HI titers were defined as the highest dilution capable of inhibiting the hemagglutination of goose's erythrocytes against 8 hemagglutination viral units.
*** The neutralizing titers were defined as the highest dilution of serum having 50% reduction of the number of viral plaques.

EXAMPLE 29. Obtaining of pAZ2

The nucleotide sequence that codifies for the amino acids from 286 to 426 of the envelope protein from the DEN-3 virus (Sequence No.39) was amplified with the oligonucleotides identified in the list of sequences as Sequence No.11 and Sequence No.13 from a DEN-3 viral strain (Osatomi K., Sumiyoshi H. Complete nucleotide sequence of dengue type 3 virus genome RNA. Virology.1990. 176(2):643-647).

The vector was created by digestion of the pM84 His plasmid with *Xba I/ Eco RI*, which contains the nucleotide sequence that codifies for the MDH protein and for a sequence of 6 histidines (Sequence No. 26). This digestion permits the insertion of the amplified

fragment by PCR within the coding region for a structural domain of the MDH protein. Upon ligation, the potential recombinants were analyzed by restriction enzyme digestion and positive clones were sequenced to check up the junctions. Competent cells MM294 were transformed with the selected clone, called pAZ2 (Fig. 8 and Sequence No.42). Upon growing the colony in LB medium, a SDS-PAGE of the cellular lysate was done. As a result a 80 kDA band was obtained, which accounted for 20% of the total cellular proteins. The size of the protein obtained corresponded to the sum of the size of the MDH protein and the DENe protein fragment from the DEN-3 virus. The protein was recognized in Immunoblotting by a HMAF anti-DEN-3 and was denominated PAZ2 (Sequence No.43).

EXAMPLE 30. Purification of the PAZ2 protein

The biomass obtained from the *E. coli* strain transformed with pAZ2 and grown at 37°C was disrupted by French press. The recombinant protein was obtained in both forms: soluble and insoluble. The protein associated to the insoluble fraction was extracted using Urea 7 M, and the supernatant, containing the PAZ2 protein, was loaded onto a G-25 column to eliminate the chaotropic agent. The fraction obtained was then loaded onto theChelating-sepharose FF column (*Pharmacia, UK*), in the presence of Cu^{++} ions. The column was washed with Imidazolee 40 mM and the protein was eluted with Imidazole 100 mM. Finally, the pure fraction was loaded onto a G-25 column to obtain the protein in the formulation (PBS). This preparation was used for immunological studies.

EXAMPLE 31. Antigenic characterization of PAZ2

The purified fraction of PAZ2 was characterized either by its recognition by different polyclonal sera and/or murine monoclonal antibodies, as well as by positive human sera to Dengue (table 36).

The highest recognitions in Dot blotting were obtained with the HMAF anti-DEN-3 (higher than those obtained with PAZ1). The recognition by HMAF against the other serotypes was lower than in the case of serotype 3. The antibodies generated by other flavivirus like the Yellow Fever virus and the Saint Louis Encephalitis virus had not any recognition at all. Finally, the reactivity against five human sera of high titers and three of low titers against DEN-3 was measured, achieving a substantial signal in both cases by Western blotting and Dot blotting.

Table 36. Reactivity of PAZ2 protein to monoclonal and polyclonal antibodies.

Abs**	*Specificity****	*PAZ2*
HMAF	DEN-1	-
HMAF	DEN-2	-
HMAF	DEN-3	+++
HMAF	DEN-4	+
HMAF	EEE	-
HMAF	YFV	-
HMAF	SLV	-
Mab 3H5	NT	-

* A total of 10 μg of purified PAZ2 was applied. The intensity of the signal obtained was evaluated from + to ++.
** HMAF were used 1:100 while the Mab 3H5 was used in dilution 1:1000.
*** EEE: Equine Encephalitis virus. YFV: Yellow Fever virus. SLV: Saint Louis Encephalitis virus. NT: Neutralizing specific-serotype

EXAMPLE 32. Characterization of the antibody response generated by PAZ2

A total of 25 Balb/c mice were i.p immunized with 20 ug of purified PAZ2 in Freund adjuvant; 10 animals were bled after four doses and the antibodies anti-DEN were evaluated by ELISA. High antibody titers against DEN-3 were obtained while, no reactivity was obtained against the rest of the serotypes (table 37 and table 40). In addition, the HI assay was done and only positive titers were found against DEN-3 (table 38 and table 40). Finally, the *in vitro* neutralization assay was done and neutralization titers of 1: 1280 against DEN-3 were obtained (table 39). No neutralization of the viral infection was found against the rest of the serotypes (table 40).

Table 37. Antibody titers against DEN-3 from the sera obtained upon immunization of mice with PAZ2.

Mouse	**Titers anti-DEN-3 PAZ2**	**Titers anti-DEN-3 PBS Control (-)**
1	>1: 128 000	< 1: 100
2	1: 128 000	< 1: 100
3	>1: 128 000	< 1: 100
4	>1: 128 000	< 1: 100
5	1: 128 000	< 1: 100
6	>1: 128 000	< 1: 100
7	1: 64000	< 1: 100
8	>1: 128 000	< 1: 100

9	1: 64000	< 1: 100
10	>1: 128 000	< 1: 100

Table 38. Titers by HI of the sera from the animals immunized with PAZ2.

Mouse	Titers anti-DEN-3 PAZ2	Titers anti-DEN-3 PBS
1	>1: 640	< 1: 5
2	1: 320	< 1: 5
3	>1: 640	< 1: 5
4	>1: 640	< 1: 5
5	>1: 640	< 1: 5
6	1: 320	< 1: 5
7	<1: 5	< 1: 5
8	1: 320	< 1: 5
9	>1: 640	< 1: 5
10	>1: 640	< 1: 5

*The HI titers were defined as the highest dilution capable of inhibiting the hemagglutination of goose's erythrocytes against 8 hemagglutination viral units.

Table 39. Viral neutralization assay with the sera of animals immunized with PAZ2.

Mouse	Neutralizing titers anti- DEN-3 PAZ2	Neutralizing titers anti-DEN-3 PBS C(-)
1	>1: 1280	< 1: 5
2	>1: 1280	< 1: 5
3	>1: 1280	< 1: 5
4	>1: 1280	< 1: 5
5	>1: 1280	< 1: 5
6	1: 640	< 1: 5
7	>1: 1280	< 1: 5
8	1: 640	< 1: 5
9	>1: 1280	< 1: 5
10	1: 640	< 1: 5

* The neutralizing titers were defined as the highest dilution of serum having 50% reduction of the number of viral plaques.

Table 40. Cross-reactivity assay against all the viral serotypes by ELISA, HI and viral neutralization with the sera of animals immunized with PAZ2.

Mixture of sera *	ELISA (anti-DEN-1)	ELISA (anti DEN-2)	ELISA (anti-DEN-3)	ELISA (anti-DEN- 4)
1 (PAZ2)	< 1/100	< 1/100	> 1: 128 000	< 1/100
2(PAZ2)	< 1/100	< 1/100	> 1: 128 000	< 1/100

Mixture of sera *	HI**anti-DEN-1	HI anti-DEN-2	HI anti-DEN-3	HI anti-DEN-4
PAZ2	< 1/ 5	< 1/ 5	>1/ 320	< 1/ 5

Mixture of sera *	Neutrallzing titers*** anti-DEN-1	Neutralizing tlters anti-DEN-2	Neutralizing titers anti-DEN-3	Neutralizing titers anti-DEN-4
1(PAZ2)	< 1: 5	< 1: 5	>1: 1280	< 1: 5
2(PAZ2)	< 1: 5	< 1: 5	>1: 1280	< 1: 5

* Each mixture was formed by three sera.
** The HI titers were defined as the highest dilution capable of inhibiting the hemagglutination of goose's erythrocytes against 8 hemagglutination viral units.
*** The neutralizing titers were defined as the highest dilution of serum having 50% reduction of the number of viral plaques.

EXAMPLE 33. Obtaining of pAZ3

The nucleotide sequence that codifies for the amino acids from 286 to 426 of the envelope protein from the DEN-3 virus (Seq. 39) was amplified with the oligonucleotides identified in the list of sequences as Sequence No.14 and Sequence No.15 from the DEN-3 viral strain (Osatomi K., Sumiyoshi H. Complete nucleotide sequence of dengue type 3 virus genome RNA. Virology.1990. 176(2):643-647).

The vector was created by digestion of the pD4 plasmid with *Bam HI/Bam HI* which contains the nucleotide sequence that codifies for the MDH protein and for a sequence of 6 histidines without stop codon (Sequence No.29). This digestion permits the fusion of the amplified fragment by PCR after the C- terminal region for the MDH protein. Upon ligation, the potential recombinants were analyzed by restriction enzyme digestion and positive clones were sequenced to check up the junctions. Competent cells W3110 were transformed with the selected clone, called pAZ3 (Fig. 9 and Sequence No.44). Upon growing the colony in LB medium, a SDS-PAGE of the cellular lysate was done. As a result a 80 kDA band was obtained, which accounted for 20% of the total cellular proteins. The size of the protein obtained corresponded to the sum of the size of the MDH protein and the DENe protein fragment from the DEN-3 virus. The protein was recognized in Immunoblotting by a HMAF anti-DEN-3 and was denominated PAZ3 (Sequence No.45).

EXAMPLE 34. Purification of the PAZ3 protein

The biomass obtained from the *E. coli* strain transformed with pAZ3 and grown at 37°C was disrupted by French press. The recombinant protein was obtained in both forms: soluble and insoluble. From the insoluble fraction, the protein was extracted using Urea 7 M, and the supernatant, containing the PAZ3 protein, was loaded onto a G-25 column to eliminate the chaotropic agent. The fraction obtained was then loaded onto the Chelating-Sepharose FF column (*Pharmacia, UK*), in the presence of Cu^{++} ions. The colum was washed with Imidazolee 45 mM and the protein was eluted with Imidazole 230 mM. Finally, the pure fraction of the protein was loaded onto a G-25 column to obtain the

protein in the formulation buffer (PBS). This preparation was used for immunological studies.

EXAMPLE 35. Antigenic characterization of PAZ3

The purified fraction of PAZ3 was characterized either by its recognition by different polyclonal sera and/or murine monoclonal antibodies, as well as by positive human sera to Dengue (table 26).

The highest recognitions in Dot blotting were obtained with the HMAF anti-DEN-3. The recognition by HMAF against the other serotypes was lower than in the case of serotype 3. The antibodies generated by other flavivirus like the Yellow Fever virus and the Saint Louis Encephalitis virus had not any recognition at all. Finally, the reactivity against three human sera of high titers and three of low titers against DEN-3 was measured, achieving a substantial signal in both cases by Dot blotting and Western blotting.

Table 41. Reactivity of PAZ3 protein to monoclonal and polyclonal antibodies.

Abs**	*Specificity****	*PAZ3*
HMAF	DEN-1	-
HMAF	DEN-2	-
HMAF	DEN-3	+++
HMAF	DEN-4	+
HMAF	EEE	-
HMAF	YFV	-
HMAF	SLV	-
Mab 3H5	NT	-

* A total of 10 μg of purified PAZ3 was applied. The intensity of the signal obtained was evaluated from + to ++.
** HMAF were used 1:100 while the Mab 3H5 was used in dilution 1:1000.
*** EEE: Equine Encephalitis virus. YFV: Yellow Fever virus. SLV: Saint Louis Encephalitis virus. NT: Neutralizing specific-serotype.

EXAMPLE 36. Characterization of the antibody response generated by PAZ3

A total of 25 Balb/c mice were i.p immunized with 20 ug of purified PAZ3 in Freund adjuvant; 10 animals were bled after four doses and the antibodies anti-DEN were evaluated by ELISA. High antibody titers against DEN-3 were obtained while, no reactivity was obtained against the rest of the serotypes (table 42 and table 45). In addition, the HI assay was done and only positive titers were found against DEN-3 (table 43 and table

45). Finally, the *in vitro* neutralization assay was done and neutralization titers of 1: 1280 against DEN-3 were obtained (table 44). No neutralization of the viral infection was found against the rest of the serotypes (table 45).

Table 42. Antibody titers against DEN-3 from the sera obtained upon immunization of mice with PAZ3.

Mouse	Titers anti-DEN-3 PAZ3	Titers anti-DEN-3 PBSControl (-)
1	>1: 128 000	< 1: 100
2	1: 128 000	< 1: 100
3	>1: 128 000	< 1: 100
4	1: 128 000	< 1: 100
5	1: 128 000	< 1: 100
6	>1: 128 000	< 1: 100
7	>1: 128 000	< 1: 100
8	1: 128 000	< 1: 100
9	1: 128 000	< 1: 100
10	>1: 128 000	< 1: 100

Table 43. Titers by HI of the sera from the animals immunized with PAZ3.

Mouse	Titers by HI* anti-DEN-3 PAZ3	Titers by HI anti-DEN-3 PBS C(-)
1	>1: 640	< 1: 5
2	>1: 640	< 1: 5
3	1: 320	< 1: 5
4	< 1: 5	< 1: 5
5	>1: 640	< 1: 5
6	< 1: 5	< 1: 5
7	1: 320	< 1: 5
8	>1: 640	< 1: 5
9	>1: 640	< 1: 5
10	1: 320	< 1: 5

*The HI titers were defined as the highest dilution capable of inhibiting the hemagglutination of goose's erythrocytes against 8 hemagglutination viral units.

Table 44. Viral neutralization assay with the sera of animals immunized with PAZ3.

Mouse	Neutralizing titers anti- DEN-3 PAZ3	Neutralizing titers anti- DEN-3 PBS C(-)
1	>1: 1280	< 1: 5
2	1: 640	< 1: 5
3	>1: 1280	< 1: 5
4	>1: 1280	< 1: 5
5	>1: 1280	< 1: 5
6	>1: 1280	< 1: 5
7	>1: 1280	< 1: 5

8	>1: 1280	< 1: 5
9	>1: 1280	< 1: 5
10	>1: 1280	< 1: 5

* The neutralizing titers were defined as the highest dilution of serum having 50% reduction of the number of viral plaques.

Table 45. Cross-reactivity assay against all the viral serotypes by ELISA, HI and viral neutralization with the sera of animals immunized with PAZ3.

Mixture of sera *	ELISA (anti-DEN-1)	ELISA (anti DEN-2)	ELISA (anti-DEN-3)	ELISA (anti-DEN- 4)
1(PAZ3)	< 1/100	< 1/100	> 1: 128 000	< 1/100
2(PAZ3)	< 1/100	< 1/100	1: 128 000	< 1/100

Mixture of sera *	HI**anti-DEN-1	HI anti-DEN-2	HI anti-DEN-3	HI anti-DEN-4
PAZ3	< 1/ 5	< 1/ 5	>1/ 320	< 1/ 5

Mixture of sera *	Neutralizing titers*** anti-DEN-1	Neutralizing titers anti-DEN-2	Neutralizing titers anti-DEN-3	Neutralizing titers anti-DEN-4
1(PAZ3)	< 1: 5	< 1: 5	>1: 1280	< 1: 5
2(PAZ3)	< 1: 5	< 1: 5	>1: 1280	< 1: 5

* Each mixture was formed by three sera.
** The HI titers were defined as the highest dilution capable of inhibiting the hemagglutination of goose's erythrocytes against 8 hemagglutination viral units.
*** The neutralizing titers were defined as the highest dilution of serum having 50% reduction of the number of viral plaques.

EXAMPLE 37. Obtaining of pID1

The nucleotide sequence that codifies for the amino acids from 286 to 426 of the envelope protein from the DEN-4 virus (Sequence No.46) was amplified with the oligonucleotides identified in the list of sequences as Sequence No.17 and Sequence No.18 from the DEN-4 virus strain genotype (Zhao B., Mackow E.R., Buckler-White A.J., Markoff L., Chancock R.M., Lai C.-J., Makino Y. Cloning full-length Dengue type 4 viral DNA sequences: Analysis of genes coding for structural proteins. Virology 1986. 155:77-88).

The vector was created by digestion of the pM108 His plasmid with *Xba I/Bam HI*, which contains the nucleotide sequence that codifies for the N-terminal region of the MDH and for a sequence of 6 histidines (Sequence No. 23). Upon ligation, the potential recombinants were analyzed by restriction enzyme digestion and positive clones were sequenced to check up the junctions. Competent cells W3110 were transformed with the selected clone, called pID1 (Fig.10 and Sequence No.47). Upon growing the colony in

Luria Bertani (LB) medium, a SDS-PAGE of the cellular lysate was done. As a result a 25 kDA band was obtained, which accounted for 10% of the total cellular proteins. The size of the protein obtained corresponded to the sum of the N-terminal region from the MDH protein and the DENe protein fragment from the DEN-4 virus. The protein was recognized in Immunoblotting by polyclonal antibodies (PA) anti-DEN-4 contained in the HMAF. This protein was denominated PID1 (Sequence No.48).

EXAMPLE 38. Purification of the protein PID1

The biomass obtained from the *E. coli* strain transformed with pID1 and grown at 37°C was disrupted by French press. The recombinant protein was obtained preponderantly as insoluble form associated to the pellet of the cellular disruption. From the pellet, the protein was extracted with urea 6 M and the supernatant, containing the PID1 protein, was loaded onto a G-25 column to eliminate the chaotropic agent. The fraction obtained was then loaded onto the Chelating-sepharose FF column (*Pharmacia, UK*), in the presence of Cu^{++} ions. The protein was eluted with Imidazolee 60 mM and the obtained volume was loaded onto a G-25 column to finally obtain the protein in the formulation buffer (PBS). This preparation was used for immunological studies.

EXAMPLE 39. Antigenic characterization of PID1

The purified fraction of PID1 was characterized either by its recognition by different polyclonal sera and/or murine monoclonal antibodies, as well as by positive human sera to Dengue (table 46).

The highest recognitions in Dot blotting were obtained with the HMAF anti-DEN-4. The recognition by HMAF against the other serotypes was lower than in the case of serotype 4. The antibodies generated by other flavivirus like the Yellow Fever virus and the Saint Louis Encephalitis virus had not any recognition at all. Finally, the reactivity against three human sera of high titers and three of low titers against DEN-4 was measured, achieving a substantial signal in both cases by Dot blotting Western blotting.

Table 46. Reactivity of PID1 protein to monoclonal and polyclonal antibodies.

Abs**	*Specificity****	*PID1*
HMAF	DEN-1	-
HMAF	DEN-2	-
HMAF	DEN-3	+
HMAF	DEN-4	++
HMAF	EEE	-

HMAF	YFV	-
HMAF	SLV	-
Mab 3H5	NT	-

* A total of 10 μg of purified PID1 was applied. The intensity of the signal obtained was evaluated from + to ++.
** HMAF were used 1:100 while the Mab 3H5 was used in dilution 1:1000.
*** EEE: Equine Encephalitis virus. YFV: Yellow Fever virus. SLV: Saint Louis Encephalitis virus. NT: Neutralizing specific-serotype.

EXAMPLE 40. Characterization of the antibody response generated by PID1

A total of 25 Balb/c mice were i.p immunized with 35 ug of purified PID1 in Freund adjuvant; 10 animals were bled after four doses and the antibodies anti-DEN were evaluated by ELISA. High antibody titers against DEN-1 were obtained while, no reactivity was obtained against the rest of the serotypes (table 47andtable 50). In addition, the HI assay was done and only positive titers were found against DEN-4 (table 48andtable 50). Finally, the *in vitro* neutralization assay was done and neutralization titers of 1:320 against DEN- 4 were obtained. However, no neutralization of the viral infection was found against the rest of the serotypes (table 49andtable 50). These results indicate the high serotype-specificity of the antibodies elicited by PID1.

Table 47. Antibody titers against DEN-4 from the sera obtained upon immunization of mice with PID1.

Mouse	Titers anti-DEN-4 PID1	Titers anti-DEN-4 PBS Control (-)
1	1/ 128 000	< 1: 100
2	1/ 128 000	< 1: 100
3	1/ 64 000	< 1: 100
4	1/ 64 000	< 1: 100
5	1/ 128 000	< 1: 100
6	1/ 32 000	< 1: 100
7	1/ 128 000	< 1: 100
8	1/ 32 000	< 1: 100
9	1/ 128 000	< 1: 100

10	1/ 128 000	< 1: 100

Table 48. Titers by HI of the sera from the animals immunized with PID1.

Mouse	Titers by HI* anti-DEN-4 PID1	Titers by HI anti-DEN-4 PBS C(-)
1	1: 320	< 1: 5
2	1: 320	< 1: 5
3	1: 640	< 1: 5
4	1:40	< 1: 5
5	< 1/ 5	< 1: 5
6	1: 320	< 1: 5
7	1: 640	< 1: 5
8	1: 640	< 1: 5
9	1:40	< 1: 5
10	1: 320	< 1: 5

*The HI titers were defined as the highest dilution capable of inhibiting the hemagglutination of goose's erythrocytes against 8 hemagglutination viral units.

Table 49. Viral neutralization assay with the sera of animals immunized with PID1.

Mouse	Neutralizing titers anti- DEN-4 PID1	Neutralizing titers anti-DEN-4 PBS C(-)
1	1: 320	< 1: 5
2	1: 80	< 1: 5
3	1: 320	< 1: 5
4	1: 320	< 1: 5
5	1: 160	< 1: 5
6	1: 320	< 1: 5
7	1: 320	< 1: 5
8	1: 320	< 1: 5
9	1: 160	< 1: 5
10	1: 40	< 1: 5

* The neutralizing titers were defined as the highest dilution of serum having 50% reduction of the number of viral plaques.

Table 50. Cross-reactivity assay against all the viral serotypes by ELISA, HI and viral neutralization with the sera of animals immunized with PID1.

Mixture of sera *	ELISA (anti-DEN-1)	ELISA (anti DEN-2)	ELISA (anti-DEN-3)	ELISA (anti-DEN- 4)
1 (PID1)	< 1/100	< 1/100	< 1/100	1: 64 000
2(PID1)	< 1/100	< 1/100	< 1/100	> 1: 128 000

Mixture of sera *	HI**anti-DEN-1	HI anti-DEN-2	HI anti-DEN-3	HI anti-DEN-4

PID1	< 1/ 5	< 1/ 5	< 1/ 5	>1:320

Mixture of sera *	Neutralizing titers*** anti-DEN-1	Neutralizing titers anti-DEN-2	Neutralizing titers anti-DEN-3	Neutralizing titers anti-DEN-4
1(PID1)	< 1: 5	< 1: 5	< 1: 5	1: 160
2(PID1)	< 1: 5	< 1: 5	< 1: 5	1: 320

* Each mixture was formed by three sera.
** The HI titers were defined as the highest dilution capable of inhibiting the hemagglutination of goose's erythrocytes against 8 hemagglutination viral units.
*** The neutralizing titers were defined as the highest dilution of serum having 50% reduction of the number of viral plaques.

EXAMPLE 41. Obtención de pID2

The nucleotide sequence that codifies for the amino acids from 286 to 426 of the envelope protein from the DEN-4 virus (Sequence No.46) was amplified with the oligonucleotides identified in the list of sequences as Sequence No.16 and Sequence No.18 from a DEN-4 viral strain (Zhao B., Mackow E.R., Buckler-White A.J., Markoff L., Chancock R.M., Lai C.-J., Makino Y. Cloning full-length Dengue type 4 viral DNA sequences: Analysis of genes coding for structural proteins. Virology 1986. 155:77-88.
The vector was created by digestion of the pM84 His plasmid with *Xba I/ Eco RI*, which contains the nucleotide sequence that codifies for the MDH protein and for a sequence of 6 histidines (Sequence No. 26). This digestion permits the insertion of the amplified fragment by PCR within the coding region for a structural domain of the MDH protein. Upon ligation, the potential recombinants were analyzed by restriction enzyme digestion and positive clones were sequenced to check up the junctions. Competent cells MM294 were transformed with the selected clone, called pID2 (Fig. 11 and Sequence No.49). Upon growing the colony in LB medium, a SDS-PAGE of the cellular lysate was done. As a result a 80 kDA band was obtained, which accounted for 20% of the total cellular proteins. The size of the protein obtained corresponded to the sum of the size of the MDH protein and the DENe protein fragment from the DEN-4 virus. The protein was recognized in Immunoblotting by a HMAF anti-DEN-4 and was denominated PID2 (Sequence No.50).

EXAMPLE 42. Purification of the PID2 protein

The biomass obtained from the *E. coli* strain transformed with pID2 and grown at 37°C was disrupted by French press. The recombinant protein was obtained in both forms: soluble and insoluble. The protein associated to the insoluble fraction was extracted using Urea 6 M, and the supernatant, containing the PID2 protein, was loaded onto a G-25 column to eliminate the chaotropic agent . The fraction obtained was then loaded onto theChelating-sepharose FF column (*Pharmacia, UK*), in the presence of Cu^{++} ions. The column was washed with Imidazolee 30 mM and the protein was eluted with Imidazole

250 mM. Finally, the pure fraction was loaded onto a G-25 column to obtain the protein in the formulation (PBS). This preparation was used for immunological studies.

EXAMPLE 43. Caracterización antigénica de PID2

The purified fraction of PID2 was characterized either by its recognition by different polyclonal sera and/or murine monoclonal antibodies, as well as by positive human sera to Dengue (table 51).

The highest recognitions in Dot blotting were obtained with the HMAF anti-DEN-4 (higher than those obtained with PID1). The recognition by HMAF against the other serotypes was lower than in the case of serotype 4. The antibodies generated by other flavivirus like the Yellow Fever virus and the Saint Louis Encephalitis virus had not any recognition at all. Finally, the reactivity against five human sera of high titers and three of low titers against DEN-3 was measured, achieving a substantial signal in both cases by Western blotting and Dot blotting.

Table 51. Reactivity of PID2 protein to monoclonal and polyclonal antibodies.

Abs**	*Specificity****	*PID2*
HMAF	DEN-1	-
HMAF	DEN-2	-
HMAF	DEN-3	+
HMAF	DEN-4	+++
HMAF	EEE	-
HMAF	YFV	-
HMAF	SLV	-
Mab 3H5	NT	-

* A total of 10 μg of purified PID2 was applied. The intensity of the signal obtained was evaluated from + to ++.
** HMAF were used 1:100 while the Mab 3H5 was used in dilution 1:1000.
*** EEE: Equine Encephalitis virus. YFV: Yellow Fever virus. SLV: Saint Louis Encephalitis virus. NT: Neutralizing specific-serotype.

EXAMPLE 44. Characterization of the antibody response generated by PID2

A total of 25 Balb/c mice were i.p immunized with 20 ug of purified PID2 in Freund adjuvant; 10 animals were bled after four doses and the antibodies anti-DEN were evaluated by ELISA. High antibody titers against DEN-4 were obtained while, no reactivity was obtained against the rest of the serotypes (table 52 and table 55). In addition, the HI

assay was done and only positive titers were found against DEN-4 (table 53 and table 55). Finally, the *in vitro* neutralization assay was done and neutralization titers of 1: 1280 against DEN-4 were obtained (table 54). No neutralization of the viral infection was found against the rest of the serotypes (table 55).

Table 52. Antibody titers against DEN-4 from the sera obtained upon immunization of mice with PID2.

Mouse	Titers anti-DEN-4 PID2	Titers anti-DEN-4 PBSControl (-)
1	1: 64000	< 1: 100
2	>1: 128 000	< 1: 100
3	1: 128 000	< 1: 100
4	>1: 128 000	< 1: 100
5	1: 64000	< 1: 100
6	>1: 128 000	< 1: 100
7	>1: 128 000	< 1: 100
8	>1: 128 000	< 1: 100
9	>1: 128 000	< 1: 100
10	1: 128 000	< 1: 100

Table 53. Titers by HI of the sera from the animals immunized with PID2.

Mouse	Titers by HI* anti-DEN-4 (PID2)	Titers by HI anti-DEN-4 PBS C(-)
1	1: 320	< 1: 5
2	1: 320	< 1: 5
3	1: 640	< 1: 5
4	1:40	< 1: 5
5	< 1/ 5	< 1: 5
6	1: 320	< 1: 5
7	1: 640	< 1: 5
8	1: 640	< 1: 5
9	1:40	< 1: 5
10	1: 320	< 1: 5

*The HI titers were defined as the highest dilution capable of inhibiting the hemagglutination of goose's erythrocytes against 8 hemagglutination viral units.

Table 54. Viral neutralization assay with the sera of animals immunized with PID2.

Mouse	Neutralizing titers anti- DEN-4 PID2	Neutralizing titers anti-DEN-4 PBS C(-)
1	>1: 1280	< 1: 5
2	>1: 1280	< 1: 5
3	>1: 1280	< 1: 5
4	>1: 1280	< 1: 5

5	1: 640	< 1: 5
6	>1: 1280	< 1: 5
7	>1: 1280	< 1: 5
8	>1: 1280	< 1: 5
9	1: 640	< 1: 5
10	>1: 1280	< 1: 5

* The neutralizing titers were defined as the highest dilution of serum having 50% reduction of the number of viral plaques.

Table 55. Cross-reactivity assay against all the viral serotypes by ELISA, HI and viral neutralization with the sera of animals immunized with PID2.

Mixture of sera *	ELISA (anti-DEN-1)	ELISA (anti DEN-2)	ELISA (anti-DEN-3)	ELISA (anti-DEN- 4)
1(PID2)	< 1/100	< 1/100	< 1/100	> 1: 128 000
2(PID2)	< 1/100	< 1/100	< 1/100	1: 64 000

Mixture of sera *	HI**anti-DEN-1	HI anti-DEN-2	HI anti-DEN-3	HI anti-DEN-4
PID2	< 1/ 5	< 1/ 5	< 1/ 5	>1/ 320

Mixture of sera *	Neutralizing titers*** anti-DEN-1	Neutralizing titers anti-DEN-2	Neutralizing titers anti-DEN-3	Neutralizing titers anti-DEN-4
1(PID2)	< 1: 5	< 1: 5	< 1: 5	>1: 1280
2(PID2)	< 1: 5	< 1: 5	< 1: 5	>1: 1280

* Each mixture was formed by three sera.
** The HI titers were defined as the highest dilution capable of inhibiting the hemagglutination of goose's erythrocytes against 8 hemagglutination viral units.
*** The neutralizing titers were defined as the highest dilution of serum having 50% reduction of the number of viral plaques.

EXAMPLE 45. Obtaining of pID3

The nucleotide sequence that codifies for the amino acids from 286 to 426 of the envelope protein from the DEN-4 virus (Sequence No.46) was amplified with the oligonucleotides identified in the list of sequences as Sequence No.19 and Sequence No.20 from the DEN-4 viral strain (Zhao B., Mackow E.R., Buckler-White A.J., Markoff L., Chancock R.M., Lai C.-J., Makino Y. Cloning full-length Dengue type 4 viral DNA sequences: Analysis of genes coding for structural proteins. Virology 1986. 155:77-88).

The vector was created by digestion of the pD4 plasmid with *Bam HI/Bam HI* which contains the nucleotide sequence that codifies for the MDH protein and for a sequence of 6 histidines without stop codon (Sequence No.29). This digestion permits the fusion of the

amplified fragment by PCR after the C- terminal region for the MDH protein. Upon ligation, the potential recombinants were analyzed by restriction enzyme digestion and positive clones were sequenced to check up the junctions. Competent cells W3110 were transformed with the selected clone, called pID3 (fig. 12 and Sequence No.51). Upon growing the colony in LB medium, a SDS-PAGE of the cellular lysate was done. As a result a 80 kDA band was obtained, which accounted for 20% of the total cellular proteins. The size of the protein obtained corresponded to the sum of the size of the MDH protein and the DENe protein fragment from the DEN-4 virus. The protein was recognized in Immunoblotting by a HMAF anti-DEN-4 and was denominated PID3 (Sequence No.52).

EXAMPLE 46. Purificación de la proteína PID3

The biomass obtained from the *E. coli* strain transformed with pID3 and grown at 37°C was disrupted by French press. The recombinant protein was obtained in both forms: soluble and insoluble. From the insoluble fraction, the protein was extracted using Urea 6 M, and the supernatant, containing the PID3 protein, was loaded onto a G-25 column to eliminate the chaotropic agent. The fraction obtained was then loaded onto the Chelating-Sepharose FF column (*Pharmacia, UK*), in the presence of Cu^{++} ions. The colum was washed with Imidazolee 45 mM and the protein was eluted with Imidazole 200 mM. Finally, the pure fraction of the protein was loaded onto a G-25 column to obtain the protein in the formulation buffer (PBS). This preparation was used for immunological studies.

EXAMPLE 47. Antigenic characterization of PID3

The purified fraction of PID3 was characterized either by its recognition by different polyclonal sera and/or murine monoclonal antibodies, as well as by positive human sera to Dengue (table 56).

The highest recognitions in Dot blotting were obtained with the HMAF anti-DEN-4. The recognition by HMAF against the other serotypes was lower than in the case of serotype 4. The antibodies generated by other flavivirus like the Yellow Fever virus and the Saint Louis Encephalitis virus had not any recognition at all. Finally, the reactivity against three human sera of high titers and three of low titers against DEN-4 was measured, achieving a substantial signal in both cases by Dot blotting and Western blotting.

Table 56. Reactivity of PID3 protein to monoclonal and polyclonal antibodies.

Abs**	*Specificity****	*PID3*
HMAF	DEN-1	-
HMAF	DEN-2	-

HMAF	DEN-3	+
HMAF	DEN-4	+++
HMAF	EEE	-
HMAF	YFV	-
HMAF	SLV	-
Mab 3H5	NT	-

* A total of 10 µg of purified PID3 was applied. The intensity of the signal obtained was evaluated from + to ++.
** HMAF were used 1:100 while the Mab 3H5 was used in dilution 1:1000.
*** EEE: Equine Encephalitis virus. YFV: Yellow Fever virus. SLV: Saint Louis Encephalitis virus. NT: Neutralizing specific-serotype.

EXAMPLE 48. Characterization of the antibody response generated by PID3

A total of 25 Balb/c mice were i.p immunized with 20 ug of purified PAZ3 in Freund adjuvant; 10 animals were bled after four doses and the antibodies anti-DEN were evaluated by ELISA. High antibody titers against DEN-4 were obtained while, no reactivity was obtained against the rest of the serotypes (table 57 and table 60). In addition, the HI assay was done and only positive titers were found against DEN-4 (table 58 and table 60). Finally, the *in vitro* neutralization assay was done and neutralization titers of 1: 1280 against DEN-4 were obtained (table 59). No neutralization of the viral infection was found against the rest of the serotypes (table 60).

Table 57. Antibody titers against DEN-4 from the sera obtained upon immunization of mice with PID3.

Mouse	Titers anti-DEN-4 (PID3)	Titers anti-DEN-4 PBSControl (-)
1	>1: 128 000	< 1: 100
2	>1: 128 000	< 1: 100
3	>1: 128 000	< 1: 100
4	1: 64 000	< 1: 100
5	1: 64 000	< 1: 100
6	>1: 128 000	< 1: 100
7	1: 128 000	< 1: 100
8	>1: 128 000	< 1: 100
9	1: 128 000	< 1: 100
10	>1: 128 000	< 1: 100

Table 58. Titers by HI of the sera from the animals immunized with PID3.

Mouse	Titers by HI* anti-DEN-4 PID3	Titers by HI anti-DEN-4 PBS C(-)
1	>1: 640	< 1: 5
2	1: 320	< 1: 5
3	>1: 640	< 1: 5
4	>1: 640	< 1: 5
5	< 1: 5	< 1: 5
6	>1: 640	< 1: 5
7	1: 320	< 1: 5
8	>1: 640	< 1: 5
9	1: 320	< 1: 5
10	>1: 640	< 1: 5

*The HI titers were defined as the highest dilution capable of inhibiting the hemagglutination of goose's erythrocytes against 8 hemagglutination viral units.

Table 59. Viral neutralization assay with the sera of animals immunized with PID3.

Mouse	Neutralizing titers* anti- DEN-4 PID3	Neutralizing titers anti-DEN-4 PBS C(-)
1	>1: 1280	< 1: 5
2	>1: 1280	< 1: 5
3	>1: 1280	< 1: 5
4	>1: 1280	< 1: 5
5	>1: 1280	< 1: 5
6	>1: 1280	< 1: 5
7	>1: 1280	< 1: 5
8	1: 640	< 1: 5
9	>1: 1280	< 1: 5
10	>1: 1280	< 1: 5

* The neutralizing titers were defined as the highest dilution of serum having 50% reduction of the number of viral plaques.

Table 60. Cross-reactivity assay against all the viral serotypes by ELISA, HI and viral neutralization with the sera of animals immunized with PID3.

Mixture of sera *	ELISA (anti-DEN-1)	ELISA (anti DEN-2)	ELISA (anti-DEN-3)	ELISA (anti-DEN- 4)
1(PID3)	< 1/100	< 1/100	< 1/100	> 1: 128 000
2(PID3)	< 1/100	< 1/100	< 1/100	1: 128 000

Mixture of sera *	HI**anti-DEN-1	HI anti-DEN-2	HI anti-DEN-3	HI anti-DEN-4
PID3	< 1/ 5	< 1/ 5	< 1/ 5	>1/ 320

Mixture of sera *	Neutralizing titers*** anti-DEN-1	Neutralizing titers anti-DEN-2	Neutralizing titers anti-DEN-3	Neutralizing titers anti-DEN-4
1(PID3)	< 1: 5	< 1: 5	< 1: 5	>1: 1280
2(PID3)	< 1: 5	< 1: 5	< 1: 5	>1/1280

* Each mixture was formed by three sera.

** The HI titers were defined as the highest dilution capable of inhibiting the hemagglutination of goose's erythrocytes against 8 hemagglutination viral units.

*** The neutralizing titers were defined as the highest dilution of serum having 50% reduction of the number of viral plaques.

EXAMPLE 49. Obtaining of pD4D2

The nucleotide sequence that codifies for the amino acids 286 to 426 of the E protein from the DEN-4 virus (Sequence No.46) was amplified with the oligonuclotides identified in the Sequences Listing as Sequence No.16 and Sequence No.21 from the viral strain of DEN-4 (Zhao B., Mackow E.R., Buckler-White A.J., Markoff L., Chancock R.M., Lai C.-J., Makino Y. Cloning full-length Dengue type 4 viral DNA sequences: Analysis of genes coding for structural proteins. Virology 1986. 155:77-88).

The vector was created by digestion *Xba /Xba I* of the pLL3 plasmid, which contains the MDH gene plus a sequence of 6 histidines in the 3' region of the gene and the sequence of the E fragment from DEN-2 in the 3' end. As result, two regions of the E protein from serotypes 2 and 4 were obtained, fused to the same MDH gene. Upon ligation, the potential recombinants were analyzed by restriction enzyme digestion and positive clones were sequenced to check up the junctions. Competent cells MM294 were transformed with the selected clone, called pD4D2 (Fig.13 and Sequence No.53). Upon growing the colony in LB medium, a SDS-PAGE of the cellular lysate was done. As a result a 110 kDa band was obtained, which accounted for 20% of the total cellular proteins. The size of the protein obtained corresponded to the sum of the MDH protein and the two fragments of the DENe protein from Dengue virus. This protein was recognized by polyclonal antibodies anti-DEN-2 and anti-DEN-4 contained in the HMAF. This protein was denominated PD4D2 (Sequence No.54).

EXAMPLE 50. Purification of the PD4D2 protein

The biomass obtained from the *E. coli* strain transformed with pD4D2 and grown at 37^{0}C was disrupted by French press. The recombinant protein was obtained either soluble or insoluble. From the pellet the protein was extracted with urea 6 M and the supernatant, containing the PD4D2 protein, was loaded onto a G-25 column to eliminate the chaotropic agent. The fraction obtained was then loaded onto the Chelating-sepharose FF column

(*Pharmacia, UK*), in the presence of Cu^{++} ions. A washing step was done with Imidazolee 30 mM and the protein was eluted with Imidazole 250 mM. Finally, the pure preparation was loaded onto a G-25 column to obtain the protein in the formulation buffer and use it for immunological studies.

EXAMPLE 51. Antigenic characterization of PD4D2

The purified fraction of PD4D2 was characterized either by its recognition by different polyclonal sera and/or murine monoclonal antibodies, as well as by positive human sera to Dengue (table 61).

The highest recognitions in Dot blotting were obtained with the HMAF anti-DEN-2 and anti-DEN-4. The recognition of the two other serotypes was less than for the case of serotypes 2 and 4. The antibodies generated by other flavivirus like the Yellow Fever virus and the Saint Louis Encephalitis virus had not any recognition at all. On the other hand, the Mab 3H5 had reactivity indeed, similar to that obtained for PLL2andPLL3. Finally, the reactivity against human sera of high and low titers against DEN-2 and DEN-4 was measured, achieving a substantial signal for both cases by Western blotting and Dot blotting.

Table 61. Reactivity of PD4D2 protein against monoclonal and polyclonal antibodies.

Abs**	*Specificity****	*PD4D2*
HMAF	DEN-1	-
HMAF	DEN-2	+++
HMAF	DEN-3	-
HMAF	DEN-4	+++
HMAF	EEE	-
HMAF	YFV	-
HMAF	SLV	-
Mab 3H5	NT	-

* A total of 10 μg of purified PD4D2 was applied. The intensity of the signal obtained was evaluated from + to ++.

** HMAF were used 1:100 while the Mab 3H5 was used in dilution 1:1000.

*** EEE: Equine Encephalitis virus. YFV: Yellow Fever virus. SLV: Saint Louis Encephalitis virus. NT: Neutralizing specific-serotype.

EXAMPLE 52. Characterization of the antibody response generated by PD4D2

A total of 25 Balb/c mice were i.p immunized with 20 ug of purified PD4D2 in Freund adjuvant; 10 animals were bled after four doses and the antibodies anti-DEN were evaluated by ELISA. High antibody titers against DEN-2 and DEN-4 were obtained while, no reactivity was obtained against the rest of the serotypes (table 62 and table 65). In addition, the hemagglutination inhibition assay (HI) was done and only positive titers were found against DEN-2 and DEN-4 (table 63 and table 65). Finally, the *in vitro* neutralization assay was done and neutralization titers of >1:1280 against DEN-2 and >1:1280 against DEN-4 were obtained (table 64). No neutralization of the viral infection was found against the rest of the serotypes (table 65).

Table 62. Antibody titers against DEN-2 and DEN-4 from the sera obtained upon immunization of mice with PD4D2.

Mouse	Titers by ELISA (PD4D2)		Titers by ELISA PBS C(-)	
	Anti-DEN-4	Anti-DEN-2	Anti-DEN-4	Anti-DEN-2
1	>1: 128 000	>1: 128 000	< 1: 100	< 1: 100
2	1: 128 000	1: 128 000	< 1: 100	< 1: 100
3	>1: 128 000	>1: 128 000	< 1: 100	< 1: 100
4	>1: 128 000	>1: 128 000	< 1: 100	< 1: 100
5	1:64 000	>1: 128 000	< 1: 100	< 1: 100
6	>1: 128 000	1: 128 000	< 1: 100	< 1: 100
7	1: 64 000	>1: 128 000	< 1: 100	< 1: 100
8	>1: 128 000	>1: 128 000	< 1: 100	< 1: 100
9	>1: 128 000	1: 128 000	< 1: 100	< 1: 100
10	1: 128 000	>1: 128 000	< 1: 100	< 1: 100

Table 63. Titers by HI of the sera from the animals immunized with PD4D2.

Mouse	Titers by HI (PD4D2)		Titers by HI PBS C(-)	
	Anti-DEN-4	Anti-DEN-2	Anti-DEN-4	Anti-DEN-2
1	>1: 640	>1: 640	< 1: 5	< 1: 5
2	>1: 640	>1: 640	< 1: 5	< 1: 5
3	>1: 640	1: 320	< 1: 5	< 1: 5
4	>1: 640	>1: 640	< 1: 5	< 1: 5
5	1: 320	1: 640	< 1: 5	< 1: 5
6	>1: 640	>1: 640	< 1: 5	< 1: 5
7	>1: 640	>1: 640	< 1: 5	< 1: 5
8	1: 320	1: 320	< 1: 5	< 1: 5
9	1: 320	1: 320	< 1: 5	< 1: 5
10	>1: 640	>1: 640	< 1: 5	< 1: 5

*The HI titers were defined as the highest dilution capable of inhibiting the hemagglutination of goose's erythrocytes against 8 hemagglutination viral units.

Table 64. Viral neutralization assay with the sera of animals immunized with PD4D2.

Mouse	Neutralizing titers* (PD4D2)		Neutralizing titers PBS C(-)	
	Anti-DEN-4	Anti-DEN-2	Anti-DEN-4	Anti-DEN-2
1	>1: 1280	>1: 1280	< 1: 5	< 1: 5
2	>1: 1280	>1: 1280	< 1: 5	< 1: 5
3	1: 1280	1: 1280	< 1: 5	< 1: 5
4	>1: 1280	>1: 1280	< 1: 5	< 1: 5
5	1: 640	1: 1280	< 1: 5	< 1: 5
6	>1: 1280	>1: 1280	< 1: 5	< 1: 5
7	>1:1280	>1:1280	< 1: 5	< 1: 5
8	1: 640	>1: 1280	< 1: 5	< 1: 5
9	>1: 1280	>1: 1280	< 1: 5	< 1: 5
10	>1: 1280	>1: 1280	< 1: 5	< 1: 5

*The neutralizing titers were defined as the highest dilution of serum having 50% reduction of the number of viral plaques.

Table 65. Cross-reactivity assay against all the viral serotypes by ELISA, HI and viral neutralization with the sera of animals immunized with PD4D2.

Mixture of sera*	ELISA (anti-DEN-1)	ELISA (anti DEN-2)	ELISA (anti-DEN-3)	ELISA (anti-DEN- 4)
1(PD4D2)	< 1/100	> 1: 128 000	< 1/100	1: 64 000
2(PD4D2)	< 1/100	> 1: 128 000	< 1/100	> 1: 128 000

Mixture of sera*	HI**anti-DEN-1	HI anti-DEN-2	HI anti-DEN-3	HI anti-DEN-4
PD4D2	< 1: 5	> 1: 320	< 1: 5	> 1: 320

Mixture of sera *	Neutralizing titers ***anti-DEN-1	Neutralizing titers anti-DEN-2	Neutralizing titers anti-DEN-3	Neutralizing titers anti-DEN-4
1(PD4D2)	< 1: 5	>1: 1280	< 1: 5	>1: 1280
2(PD4D2)	< 1: 5	>1: 1280	< 1: 5	>1: 1280

* Each mixture was formed by three sera.
** The HI titers were defined as the highest dilution capable of inhibiting the hemagglutination of goose's erythrocytes against 8 hemagglutination viral units.
*** The neutralizing titers were defined as the highest dilution of serum having 50% reduction of the number of viral plaques.

EXAMPLE 53. Protection assay

For the evaluation of the protection conferred to mice immunized with all the assayed variants upon challenge with homologous lethal DEN, 15 mice of each group were used. Each animal received one dose of 100 LD_{50} of lethal DEN by intracraneal inoculation and they were observed during 21 days to study the percentage of lethality. As positive controls, groups of 15 mice immunized with the four viral preparations (DEN-1, DEN-2, DEN-3 and DEN-4) were used. All the mice of these control groups survived while mice from the negative control group become sick between 7-11 days after challenge; therefore, achieving a 100% of mortality. Finally, groups immunized with the fusion proteins under study had between an 80% and 100% of protection and in all the cases, significant differences with respect to the control group were found (table 66).

Table 66. Percentage of survival in mice immunized with the protein variants assayed upon challenge with the homologous lethal Dengue virus.

Immunogen	Percentage of survival*
PBS	0
DEN-1	100
DEN-2	100
DEN-3	100
DEN-4	100
PLL1	86
PLL2	100
PLL3	100
PLH1	80
PLH2	100
PLH3	100
PAZ1	80
PAZ2	100
PAZ3	100
PID1	86
PID2	100
PID3	100
PD4D2	100 (DEN-4) 100 (DEN-2)

* It was calculated: (# of mice survived)/ (# total of mice). Data of survivors were taken 21 after challenge. In the case of mice immunized with pD4D2, 15 were challenged with DEN-4 and 15 with DEN-2.

EXAMPLE 54. Lymphoproliferative response

Animals from different groups immunized with the chimeric proteins containing the E fragment from DEN-2 (PLL1, PLL2 and PLL3), and a placebo group, were sacrified 15 days after the last dose. Then, the spleen of the animals was harvested and the lymphoproliferative response against the four serotypes of Dengue virus was studied. Table 67 shows the results of the stimulation indexes obtained, which demonstrate that a serotype specific response was achieved.

Table 67. Stimulation indexes, against the four Dengue viral serotypes, of lymphocytes from mice immunized with PLL1, PLL2 and PLL3.

	PLL1	**PLL2**	**PLL3**	**C(-)**
DEN-1	1.3 *	1.0	0.8	1.2
DEN-2	**12.5**	**10.3**	**8.9**	1.4
DEN-3	1.0	1.6	1.8	1.4
DEN-4	1.7	1.5	1.7	1.1
Control Antigen	1.1	1.0	1.3	0.9
PHA**	**13.3**	**16.5**	**11.1**	**12.0**

*Stimulation index: cocient of the counts per minute of the samples between the counts per minute of the control of the spontaneous synthesis of DNA.
** mitogen: Phytohemagglutinin.

CLAIMS

1. Chimeric nucleotide chains characterized by being the specific combination of sequences corresponding to fragments of the E protein from Flavivirus, with sequences corresponding to the codifying gene for the mutated MDH protein, identified in the Sequences Listing as Sequence No.26, Sequence No.29 or with the fragment of these identified as Sequence No.23, which codify for chimeric proteins capable of inducing a immune response of neutralizing and protective antibodies in the recipient, against the Flavivirus, when these proteins are present in a pharmaceutical preparation.
2. Chimeric nucleotide chains according to claim 1, characterized by being the specific combination of one or several sequences that codify for the amino acids 286 to 426 of the E protein from Dengue viruses with the nucleotide sequence of the MDH protein, where these sequences are inserted and/or fused to the sequences identified in the Sequences Listing as Sequence No.26, Sequence No.29 or Sequence No.23, which codify for chimeric proteins capable of inducing a humoral immune response of neutralizing and protective antibodies in the recipient, serotype-specific against each serotype of Dengue virus.
3. Chimeric nucleotide chains according to claims 1 and 2, characterized by being formed by the codifying sequences for the amino acids 286 to 426 of the E protein from each serotype 1, 2, 3 and 4 of Dengue virus, fused to the 3' end region that codifies for the first 45 amino acids of the MDH protein, identified as Sequence No.23.
4. Chimeric nucleotide chains according to claims 1 and 2, characterized by being formed by the codifying sequences for the amino acids 286 to 426 of the E protein from each serotype 1, 2, 3 and 4 of Dengue virus, inserted in the region that codifies for the amino acid 45 of the MDH protein, identified as Sequence No.26.
5. Chimeric nucleotide chains according to claims 1 and 2, characterized by being formed by the codifying sequences for the amino acids 286 to 426 of the E protein from each serotype 1, 2, 3 and 4 of Dengue virus, fused to the 3' end region that codifies for the MDH protein, identified as Sequence No.29.
6. Chimeric nucleotide chains according to claims 1 and 2, characterized by being the combination of the nucleotide sequences that codify for the amino acids 286 to 426 of the E protein from two different serotypes of Dengue virus, with the sequence of the MDH, where one of the sequences corresponding to the E protein is inserted in the region that codifies for the amino acid 45 of the MDH

protein and the other is fused to the 3' region of the same MDH sequence, identified as sequence No.29.

7. Recombinant chimeric proteins characterized by being the expression products of the nucleotide chains from claims 1, 2, 3, 4, 5 and 6, and being capable of inducing a humoral immune response of neutralizing and protective antibodies in the recipient, serotype-specific only against each homologous serotype of Dengue virus, when these proteins are present in a pharmaceutical preparation.
8. Chimeric recombinant proteins characterized by being the expression products of the nucleotide chains from claims 1 and 6, and being capable of inducing a humoral immune response, neutralizing and protective antibodies in the recipient, specific against the two homologous serotypes of Dengue virus.
9. Chimeric nucleotide chains, identified essentially by the Sequence No.24, according to claims 1, 2 and 3 characterized by being the combination of the sequences identified in the Sequences Listing as Sequence No.22 and Sequence No.23, which codifies for the chimeric protein PLL1.
10. Chimeric protein PLL1, according to claims 1, 2 and 7 characterized by having essentially the amino acid sequence identified in the Sequences Listing as Sequence No.25, be the expression product of the Sequence No.24 and being capable of inducing a humoral response, neutralizing and protective in the recipient specific against the Dengue-2 virus, when PLL1 is present in a pharmaceutical preparation.
11. Chimeric nucleotide chains according to claims 1, 2 and 4 identified essentially as Sequence No.27, characterised by being the combination of the sequences identified in the Sequences Listing as Sequence No.22 and Sequence No.26, which codifies for the chimeric protein PLL2.
12. Chimeric protein PLL2, according to claims 1, 2 and 7 characterized by having essentially the amino acid sequence identified in the Sequences Listing as Sequence No.28, be the expression product of the Sequence No. 27 and being capable of inducing a humoral response, neutralizing and protective in the recipient specific against the Dengue-2 virus, when PLL2 is present in a pharmaceutical preparation.
13. Chimeric nucleotide chains according to claims 1, 2 and 5 identified essentially as Sequence No.30, characterised by being the combination of the sequences identified in the Sequences Listing as Sequence No.22 and Sequence No.29, which codifies for the chimeric protein PLL3.
14. Chimeric protein PLL3, according to claims 1, 2 and 7 characterized by having essentially the amino acid sequence identified in the Sequences Listing as

Sequence No.31, be the expression product of the Sequence No.30 and being capable of inducing a humoral response, neutralizing and protective in the recipient specific against the Dengue-2 virus, when PLL3 is present in a pharmaceutical preparation.

15. Chimeric nucleotide chains according to claims 1, 2 and 3 identified essentially as Sequence No.33, characterised by being the combination of the sequences identified in the Sequences Listing as Sequence No.23 and Sequence No.32, which codifies for the chimeric protein PLH1.

16. Chimeric protein PLH1, according to claims 1, 2 and 7 characterized by having essentially the amino acid sequence identified in the Sequences Listing as Sequence No.34, be the expression product of the Sequence No.33 and being capable of inducing a humoral response, neutralizing and protective in the recipient specific against the Dengue-1 virus, when PLH1 is present in a pharmaceutical preparation.

17. Chimeric nucleotide chains according to claims 1, 2 and 4 identified essentially as Sequence No.35, characterised by being the combination of the sequences identified in the Sequences Listing as Sequence No.26 and Sequence No.32, which codifies for the chimeric protein PLH2.

18. Chimeric protein PLH2, according to claims 1, 2 and 7 characterized by having essentially the amino acid sequence identified in the Sequences Listing as Sequence No.36, be the expression product of the Sequence No.35 and being capable of inducing a humoral response, neutralizing and protective in the recipient specific against the Dengue-1 virus, when PLH2 is present in a pharmaceutical preparation.

19. Chimeric nucleotide chains according to claims 1, 2 and 5 identified essentially as Sequence No.37, characterised by being the combination of the sequences identified in the Sequences Listing as Sequence No.29 and Sequence No.32, which codifies for the chimeric protein PLH3.

20. Chimeric protein PLH3, according to claims 1, 2 and 7 characterized by having essentially the amino acid sequence identified in the Sequences Listing as Sequence No.38, be the expression product of the Sequence No.37 and being capable of inducing a humoral response, neutralizing and protective in the recipient specific against the Dengue-1 virus, when PLH3 is present in a pharmaceutical preparation.

21. Chimeric nucleotide chains according to claims 1, 2 and 3 identified essentially as Sequence No.40, characterised by being the combination of the sequences

identified in the Sequences Listing as Sequence No.23 and Sequence No.39, which codifies for the chimeric protein PAZ1.

22. Chimeric protein PAZ1, according to claims 1, 2 and 7 characterized by having essentially the amino acid sequence identified in the Sequences Listing as Sequence No.41, be the expression product of the Sequence No.40 and being capable of inducing a humoral response, neutralizing and protective in the recipient specific against the Dengue-3 virus, when PAZ1 is present in a pharmaceutical preparation.

23. Chimeric nucleotide chains according to claims 1, 2 and 4 identified essentially as Sequence No.42, characterised by being the combination of the sequences identified in the Sequences Listing as Sequence No.26 and Sequence No.39, which codifies for the chimeric protein PAZ2.

24. Chimeric protein PAZ2, according to claims 1, 2 and 7 characterized by having essentially the amino acid sequence identified in the Sequences Listing as Sequence No.43, be the expression product of the Sequence No.42 and being capable of inducing a humoral response, neutralizing and protective in the recipient specific against the Dengue-3 virus, when PAZ2 is present in a pharmaceutical preparation.

25. Chimeric nucleotide chains according to claims 1, 2 and 5 identified essentially as Sequence No.44, characterised by being the combination of the sequences identified in the Sequences Listing as Sequence No.29 and Sequence No.39, which codifies for the chimeric protein PAZ3.

26. Chimeric protein PAZ3, according to claims 1, 2 and 7 characterized by having essentially the amino acid sequence identified in the Sequences Listing as Sequence No.45, be the expression product of the Sequence No.44 and being capable of inducing a humoral response, neutralizing and protective in the recipient specific against the Dengue-3 virus when, PAZ3 is present in a pharmaceutical preparation.

27. Chimeric nucleotide chains according to claims 1, 2 and 3 identified essentially as Sequence No.47, characterised by being the combination of the sequences identified in the Sequences Listing as Sequence No.23 and Sequence No.46, which codifies for the chimeric protein PID1.

28. Chimeric protein PID1, according to claims 1, 2 and 7 characterized by having essentially the amino acid sequence identified in the Sequences Listing as Sequence No.48, be the expression product of the Sequence No.47 and being capable of inducing a humoral response, neutralizing and protective in the

recipient specific against the Dengue-4 virus, when PID1 is present in a pharmaceutical preparation.

29. Chimeric nucleotide chains according to claims 1, 2 and 4 identified essentially as Sequence No.49, characterised by being the combination of the sequences identified in the Sequences Listing as Sequence No.26 and Sequence No.46, which codifies for the chimeric protein PID2.

30. Chimeric protein PID2, according to claims 1, 2 and 7 characterized by having essentially the amino acid sequence identified in the Sequences Listing as Sequence No.50 be the expression product of the Sequence No.49 and being capable of inducing a humoral response, neutralizing and protective in the recipient specific against the Dengue-4 virus, when PID2 is present in a pharmaceutical preparation.

31. Chimeric nucleotide chains according to claims 1, 2 and 5 identified essentially as Sequence No.51, characterised by being the combination of the sequences identified in the Sequences Listing as Sequence No.29 and Sequence No.46, which codifies for the chimeric protein PID3.

32. Chimeric protein PID3, according to claims 1, 2 and 7 characterized by having essentially the amino acid sequence identified in the Sequences Listing as Sequence No.52 be the expression product of the Sequence No.51 and being capable of inducing a humoral response, neutralizing and protective in the recipient specific against the Dengue-4 virus, when PID3 is present in a pharmaceutical preparation.

33. A pharmaceutical preparation capable of inducing in the recipient a protective immune response, specific against the Dengue virus, according to claim 1, characterized by having two or more of the chimeric proteins described in claims 1, 2, 7, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30 and 32, and a pharmacological vehicle.

34. A pharmaceutical preparation according to claim 33, characterized by being a preventive or therapeutic agent against the Dengue viruses, for oral, intramuscular, subcutaneous, mucosal or intravenous use.

35. A diagnostic mean characterized by having one or several of the proteins described in claims 1, 2, 7, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30 and 32, useful for the diagnosis and serotyping of the Dengue virus.