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(54) Title: IMMUNISATION METHOD AGAINST THE 4 DENGUE SEROTYPES

(54) Titre : METHODE D'IMMUNISATION CONTRE LES 4 SEROTYPES DE LA DENGUE

(57) Abstract: The invention relates to a method for inducing a protection against the 4 dengue serotypes in a patient, that comprises: (a) a first series of administrations (i) of a dose of a dengue vaccinal virus of a first serotype and a dose of a dengue vaccinal virus of a second serotype, and (ii) of a dose of a dengue vaccinal virus of a third serotype and a dose of a dengue vaccinal virus of a fourth serotype, and (b) a second series of administrations of the (i) and (ii) doses, wherein the (i) and (ii) doses are administered simultaneously at separate anatomical sites and wherein the second series is administered at least 30 days and at most 12 months after the first series.

(57) Abrégé : L'invention concerne une méthode pour induire une protection contre les 4 sérotypes de la dengue chez un patient, comprenant : (a) une première série d'administrations (i) d'une dose d'un virus vaccinal de la dengue d'un premier sérototype et d'une dose d'un virus vaccinal de la dengue d'un deuxième sérototype, et (ii) d'une dose d'un virus vaccinal de la dengue d'un troisième sérototype et d'une dose d'un virus vaccinal de la dengue d'un quatrième sérototype et (b) une deuxième série d'administrations des doses (i) et (ii), dans laquelle les doses (i) et (ii) sont administrées simultanément à des sites anatomiques distincts, et dans laquelle la deuxième série est mise en œuvre au moins 30 jours à au plus 12 mois après la première série.

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IMMUNIZATION METHOD AGAINST THE 4 DENGUE SEROTYPES

The invention relates to a method for inducing a protection against the 4 dengue serotypes in a patient, comprising

5 (a) a first series of administrations (i) of a dose of a vaccinal dengue virus of a first serotype and of a dose of a vaccinal dengue virus of a second serotype, and (ii) of a dose of a vaccinal dengue virus of a third serotype and of a dose of a vaccinal dengue virus of a fourth serotype, and

10 (b) a second series of administrations of doses (i) and (ii), in which the doses (i) and (ii) are administered simultaneously at separate anatomical sites, and

15 in which the second series (b) is implemented at least 30 days to at most 12 months after the first series (a).

Dengue diseases are caused by four viruses of the flavivirus genus, of 20 the serological type, which are similar but distinct from an antigenic point of view (Gubler et al., 1988 In: Epidemiology of arthropod-borne viral disease. Monath TPM, editor, Boca Raton (FL): CRC Press: 223-60; Kautner et al., 1997, J. of Pediatrics, 131:516-524; Rigau-Pérez et al., 1998, Lancet; 352: 971-977; Vaughn et al., 1997, J Infect Dis; 176: 322-30). Infection with a dengue 25 serotype can produce a clinical disease spectrum ranging from a nonspecific viral syndrome to a severe hemorrhagic disease which is fatal. The incubation period of dengue fever after a mosquito bite is approximately 4 days (ranging from 3 to 14 days). Dengue fever is characterized by a biphasic fever, headaches, pain in various parts of the body, prostration, eruptions, 30 lymphadenopathy and leukopenia (Kautner et al., 1997, J. of Pediatrics, 131:516-524; Rigau-Pérez et al., 1998, Lancet; 352: 971-977). The viremia period is the same as the febrile period (Vaughn et al., 1997, J. Infect. Dis.; 176: 322-30). Recovery from dengue fever occurs after 7 to 10 days, but there is usually a prolonged asthenia. Decreases in leukocyte and platelet count are common.

Hemorrhagic dengue is a severe febrile disease characterized by anomalies in homeostasis and an increase in vascular permeability which can result in hypovolemia and in hypotension (dengue with shock syndrome) often

complicated by severe internal hemorrhaging. The mortality rate of hemorrhagic dengue can be up to 10% without treatment, but is 1% in most centers with experience in treatment (WHO technical Guide, 1986. *Dengue haemorrhagic fever: diagnosis, treatment and control*, p1-2. World Health Organization, Geneva, Switzerland).

The routine laboratory diagnosis of dengue is based on isolation of the virus and/or detection of antibodies specific for the dengue virus.

Dengue is the second most common tropical infectious disease after malaria, more than half the world's population living in regions where there is a risk of epidemic transmission. Each year, cases of dengue are estimated at 50-100 million, cases of patients hospitalized for hemorrhagic dengue at 500 000, and the number of deaths at 25 000. Dengue is endemic in Asia, in the Pacific region, in Africa, in Latin America and in the Caribbean. More than 100 tropical countries are endemic for dengue virus infections and hemorrhagic dengue has been documented in 60 of these countries (Gubler, 2002, *TRENDS in Microbiology*. 10:100-103; Monath, 1994, *Proc. Natl. Acad. Sci.*; 91: 2395-2400). A certain number of well-described factors appear to be involved in dengue: population growth; unplanned and uncontrolled urbanization, in particular in combination with poverty; an increase in air travel; the lack of effective control of mosquitoes and the deterioration of hygiene infrastructures and of public health (Gubler, 2002, *TRENDS in Microbiology*. 10:100-103). Individuals who travel and expatriates are increasingly warned about dengue (Shirtcliffe et al., 1998, *J. Roy. Coll. Phys. Lond.*; 32: 235-237). Dengue has constituted one of the main causes of febrile diseases in American troops during deployments in tropical zones endemic for dengue (DeFraites et al., 1994, *MMWR* 1994; 43: 845-848).

The viruses are maintained in a cycle which involves humans and *Aedes aegypti*, a domestic mosquito which bites during the day, and which prefers to feed off humans. The infection in humans is initiated by injection of the virus while an infected *Aedes aegypti* mosquito feeds on the blood. The virus in the saliva is deposited mainly in the extravascular tissues. The first category of cells infected after inoculation are dendritic cells, which then migrate to the lymph nodes (Wu et al., 2000, *Nature Med.*; 7:816-820). After an initial

replication in the skin and in the lymph nodes, the virus appears in the blood during the acute febrile phase, generally for 3 to 5 days.

Monocytes and macrophages are, with dendritic cells, among the first targets of the dengue virus. Protection against a homotypic reinfection is 5 complete and probably lasts for a lifetime, but crossprotection between the various dengue types lasts less than a few weeks to a few months (Sabin, 1952, Am. J. Trop. Med. Hyg.; 1: 30-50). Consequently, an individual can experience an infection with a different serotype. A second infection with dengue is in theory a risk factor for developing a severe dengue disease. 10 However, hemorrhagic dengue is multifactorial: these factors include the strain of the virus involved, and also the age, the immune status and the genetic predisposition of the patient. Two factors play a major role in the occurrence of hemorrhagic dengue: rapid viral replication with a high viremia (the severity of the disease being associated with the level of viremia; Vaughn et al., 2000, J. 15 Inf. Dis.; 181: 2-9) and a substantially inflammatory response with the release of high levels of inflammatory mediators (Rothman and Ennis, 1999, Virology; 257: 1-6). There is no specific treatment against dengue. The treatment for dengue fever is symptomatic with confinement to bed, control of the fever and of the pain with antipyretics and analgesics, and adequate fluid intake. The 20 treatment for hemorrhagic dengue requires equilibration of fluid losses, replacement of clotting factors and heparin infusion.

Preventive measures are currently based on controlling the vector and taking personal protection steps which are difficult to implement and expensive. No vaccine against dengue has been approved at this time. Given that the four 25 dengue serotypes are in circulation in the world and since they have been reported as being involved in cases of dengue hemorrhagic fever, immunization should ideally confer protection against the four serotypes of the dengue virus.

The use of different anatomical sites for the administration of dengue virus has already been described in the literature.

30 Thus, Halstead et al. (1973, Am. J. Trop. Med. Hyg., 22:375-381) have shown that an administration of wild-type dengue viruses carried out at 2 or 4 separate anatomical sites for the 4 different serotypes induces protection against a subsequent infection. However, the authors observe no superiority of

this type of separate immunization compared with an immunization carried out at a single site.

Attenuated viral forms of dengue viruses result in interferences in humans when they are administered in the form of a tetravalent vaccine. This 5 phenomenon has in particular been described in the following publications: Gubler D.J. Clin. Microbiol. Rev. 1998; 11 (3):480-96; Rothman A.L. et al Vaccine 2001; 19:4694-9.

10 Zhou H and Deem MW. (Vaccine. 2006 Mar 24;24(14):2451-9) have developed a mathematical model based only on the use of the CD8 epitopes and aimed at simulating the interferences between the CD8 epitopes of the 4 dengue serotypes. According to this theoretical model, the best way of avoiding the interferences would be to carry out a primary immunization using a non-dominant CD8 epitope, followed by a booster by means of an administration at different anatomical sites of the same CD8 epitopes of each of the 4 serotypes.

15 There exists, therefore, a need for a method for reducing the interferences between the various serotypes and for inducing neutralizing antibodies against the 4 dengue serotypes.

20 The inventors have demonstrated that it is possible to generate a homologous immune response comprising antibodies that neutralize the 4 serotypes where the latter are administered simultaneously in pairs at separate anatomical sites in a first series of administrations and then in a second series of administrations implemented 30 days to 12 months after the first administration of the 4 serotypes.

25 The inventors have in particular shown that a DEN-1,2 bivalent immunization concomitant with a DEN-3,4 bivalent immunization, carried out at two separate anatomical sites and followed by a booster of the same vaccinal doses under the same conditions, induces high responses against the four serotypes in all the monkeys immunized with the exception of one serotype in one animal. Conversely, a tetravalent immunization carried out at a single site 30 made it possible to induce a satisfactory response only against two serotypes out of 4.

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The immune response generated by the method according to the present invention is therefore quantitatively and qualitatively greater (covers all the serotypes).

According to a first subject, the present invention therefore relates to a
5 method for inducing a homologous protection against the 4 dengue serotypes in a patient, comprising

(a) a first series of administrations (i) of a dose of a vaccinal dengue virus of a first serotype and of a dose of a vaccinal dengue virus of a second serotype, and (ii) of a dose of a vaccinal dengue virus of a third serotype and of a dose of a
10 vaccinal dengue virus of a fourth serotype, and

(b) a second series of administrations of doses (i) and (ii), in which the doses (i) and (ii) are administered simultaneously at separate anatomical sites, and

in which the second series is implemented at least 30 days and at most 12
15 months after the first series, and

in which said vaccinal dengue viruses of a first, a second, a third and a fourth serotype are respectively:

vaccinal dengue viruses of serotypes 1, 2, 3 and 4;

vaccinal dengue viruses of serotypes 1, 3, 2 and 4;

20 vaccinal dengue viruses of serotypes 1, 4, 2 and 3;

vaccinal dengue viruses of serotypes 2, 3, 1 and 4;

vaccinal dengue viruses of serotypes 2, 4, 1 and 3; or

vaccinal dengue viruses of serotypes 3, 4, 1 and 2.

According to another embodiment of the method according to the invention,
25 the vaccinal dengue viruses (i) are administered in the form of a single bivalent vaccinal dose.

According to another embodiment of the method according to the invention, the vaccinal dengue viruses (ii) are administered in the form of a single bivalent vaccinal dose.

According to one specific embodiment of the immunization method according to the invention, said vaccinal dengue virus serotype 1 is selected from the group consisting of the VDV1 strain and of a ChimeriVax™ DEN-1.

5 According to another specific embodiment of the method according to the invention, said vaccinal dengue virus serotype 2 is selected from the group consisting of the VDV2 strain and of a ChimeriVax™ DEN-2.

According to another specific embodiment of the method according to the invention, said vaccinal dengue virus serotype 1 is the VDV1 strain and said vaccinal dengue virus serotype 2 is the VDV2 strain.

10 According to another specific embodiment of the method according to the invention, said vaccinal dengue virus serotype 1 is a ChimeriVax™ DEN-1 and said vaccinal dengue virus serotype 2 is a ChimeriVax™ DEN-2.

According to another specific embodiment of the method according to the invention, said vaccinal dengue virus serotype 3 is a ChimeriVax™ DEN-3.

15 According to another specific embodiment of the method according to the invention, said vaccinal dengue virus serotype 4 is a ChimeriVax™ DEN-4.

According to another specific embodiment of the method according to the invention, the first and second serotypes are, respectively, CYD DEN-1 and CYD DEN-2 and the third and fourth serotypes are, respectively, CYD DEN-3 and CYD 20 DEN-4.

According to another specific embodiment of the method according to the invention, the first and second serotypes are, respectively, CYD DEN-1 and CYD DEN-3 and the third and fourth serotypes are, respectively, CYD DEN-2 and CYD DEN-4.

25 According to another specific embodiment of the method according to the invention, the amount of vaccinal dengue viruses serotypes 1, 2, 3 and 4 is within a range of from 10^3 to 10^6 CCID₅₀.

According to another embodiment of the method according to the invention, the vaccinal viruses used in the second series of administrations are identical to 30 those used in the first series of administrations.

According to another embodiment of the method according to the invention, the second series of administrations is implemented 30 to 60 days after the first series of administrations.

5 A subject of the present invention is also a kit when used for immunization against the dengue virus, comprising a case containing at least the vaccinal dengue viruses serotypes 1, 2, 3 and 4

(a) in the form of monovalent compositions containing 4 separate containers, or

10 (b) in the form of two bivalent compositions containing 2 separate containers.

According to one embodiment, the kit according to the invention comprises at least:

(a) a first container containing a bivalent vaccine comprising a ChimeriVax™ DEN-1 and a ChimeriVax™ DEN-2, and

15 (b) a second container containing a bivalent vaccine comprising a ChimeriVax™ DEN-3 and a ChimeriVax™ DEN-4.

According to another embodiment, the kit according to the invention comprises at least:

20 (a) a first container containing a bivalent vaccine comprising a ChimeriVax™ DEN-1 and a ChimeriVax™ DEN-3, and

(b) a second container containing a bivalent vaccine comprising a ChimeriVax™ DEN-2 and a ChimeriVax™ DEN-4.

25 A subject of the present invention is also a kit for immunization against the dengue virus, comprising a case containing at least the vaccinal dengue viruses of a first serotype and of a second serotype,

(a) in the form of 2 monovalent compositions contained in 2 separate containers, or

(b) in the form of a bivalent composition contained in 1 single container.

According to one embodiment, the kit comprises at least:

30 (a) a container containing a bivalent vaccine comprising a ChimeriVax™ DEN-1 and a ChimeriVax™ DEN-3, or

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(b) a container containing a bivalent vaccine comprising a ChimeriVax™ DEN-2 and a ChimeriVax™ DEN-4, or

(c) a container containing a bivalent vaccine comprising a ChimeriVax™ DEN-1 and a ChimeriVax™ DEN-2, or

5 (d) a container containing a bivalent vaccine comprising a ChimeriVax™ DEN-3 and a ChimeriVax™ DEN-4.

According to another embodiment, there is provided a kit when used for immunization against the dengue virus, comprising a case containing at least the vaccinal dengue viruses of serotypes 1, 2, 3 and 4

10 (a) in the form of monovalent compositions contained in 4 separate containers, or

(b) in the form of two bivalent compositions contained in 2 separate containers;

15 wherein said monovalent compositions or said bivalent compositions are used in a method for inducing a protection against the 4 dengue serotypes 1-4 in a patient, comprising:

20 a first series of administrations (i) of a dose of a vaccinal dengue virus of a first serotype and of a dose of a vaccinal dengue virus of a second serotype, and (ii) of a dose of a vaccinal dengue virus of a third serotype and of a dose of a vaccinal dengue virus of a fourth serotype; and

a second series of administrations of doses (i) and (ii), in which the doses (i) and (ii) are administered simultaneously at separate anatomical sites, and

in which the second series is implemented at least 30 days to at most 12 months after the first series, and

25 in which said vaccinal dengue viruses of a first, a second, a third and a fourth serotype are respectively:

vaccinal dengue viruses of serotypes 1, 2, 3 and 4;

vaccinal dengue viruses of serotypes 1, 3, 2 and 4;

vaccinal dengue viruses of serotypes 1, 4, 2 and 3;

30 vaccinal dengue viruses of serotypes 2, 3, 1 and 4;

vaccinal dengue viruses of serotypes 2, 4, 1 and 3; or

vaccinal dengue viruses of serotypes 3, 4, 1 and 2.

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The present invention also provides a bivalent composition or a bivalent vaccine comprising an immunoeffective amount of the dengue vaccinal viruses of a first serotype and of a second serotype and a pharmaceutically acceptable excipient.

5 According to a specific embodiment, the bivalent composition or vaccine comprises the vaccinal viruses selected from the group consisting of: ChimeriVax™ DEN-1 and ChimeriVax™ DEN-3; or ChimeriVax™ DEN-2 and ChimeriVax™ DEN-4; or ChimeriVax™ DEN-1 and a ChimeriVax™ DEN-2; or ChimeriVax™ DEN-3 and ChimeriVax™ DEN-4.

The invention will be described in further detail in the description which follows.

Definitions

In the context of the present invention, two anatomical sites are 5 "separate" if they are drained by different lymph nodes. For example, the right arm and the left arm are considered to be separate sites. The following separate sites may also be mentioned by way of nonlimiting examples: right arm/right thigh; left arm/left thigh, left arm/right thigh.

In the context of the present invention, the term "simultaneous 10 administrations" is intended to mean administrations implemented on the same day (i.e. at most 24 h). Simultaneous administrations are advantageously carried out at most 1 hour apart, conventionally 1-5 minutes apart.

In the context of the present invention, the doses (i) are administered at a first anatomical site, either in the form of two monovalent doses or in the form 15 of a single bivalent dose. The doses (ii) are, for their part, administered simultaneously at a second anatomical site, either in the form of two monovalent doses or in the form of a single bivalent dose, the first and second sites being separate sites as defined above.

"Dengue viruses" or "DENs" are positive, single-stranded RNA viruses 20 belonging to the Flavivirus genus of the *flaviviridae* family. The genomic RNA contains a type I cap at the 5' end but lacks a poly-A tail at the 3' end. The genomic organization consists of the following elements: 5' noncoding region (NCR), structural proteins (capsid (C), premembrane/membrane (prM/M), envelope (E)) and nonstructural proteins (NS1-NS2A-NS2B-NS3-NS4A-NS4B-25 NS5), and 3' NCR. The genomic viral RNA is associated with the capsid proteins so as to form a nucleocapsid. As for the other flaviviruses, the DEN viral genome encodes an uninterrupted coding region which is translated into a single polyprotein.

In the context of the present invention, the term "vaccinal dengue virus" 30 is intended to mean any viral form of the dengue virus capable of inducing a specific homologous immune response, preferably any viral form of the dengue virus that can be used in the context of an immunization program in humans against dengue virus infection. The term "vaccinal dengue viruses" is therefore

intended to mean inactivated viruses, attenuated viruses, or recombinant proteins such as the dengue virus envelope protein.

A vaccinal virus is considered to be "inactivated" if it no longer replicates on permissive cells.

5 A vaccinal virus is considered to be "attenuated" if, after growth at 37°C or 39°C on Huh-7, VERO and/or C6/36 hepatic cells, said vaccinal virus has a maximum titer that is at least 10-fold less than the maximum titer obtained with the wild-type parental strain under the same culture conditions and as measured using the tittering method. A vaccinal virus that exhibits decreased
10 growth on at least one of the three cell types identified above is therefore considered to be "attenuated" in the context of the present invention.

A vaccinal virus that can be used in humans has a positive benefit/risk ratio, said ratio generally making it possible to comply with the regulatory requirements for obtaining a marketing authorization. A vaccinal dengue virus
15 used in the context of the present invention is preferably an attenuated virus such that it does not induce the disease in humans. Advantageously, said vaccinal virus produces only side effects that are at most of moderate intensity (i.e. moderate to weak, or even zero) in the majority of the individuals immunized, while at the same time conserving its ability to induce a neutralizing
20 antibody response.

By way of nonlimiting examples of vaccinal dengue virus that can be used in the context of the present invention, mention may be made of: inactivated vaccinal viruses, attenuated vaccinal viruses such as the attenuated strains VDV-1 or VDV-2, the strains described, for example, in applications:
25 WO02/66621, WO0057904, WO0057908, WO0057909; WO0057910, WO02/0950075 and WO02/102828, or chimeras. Chimeric viruses have the particularity of having the characteristics of the attenuated viruses as defined above. Any chimeric virus expressing the dengue virus envelope protein and inducing an immune response comprising antibodies that neutralize the
30 serotype from which the envelope protein is derived can therefore be used in the context of the present invention. By way of nonlimiting examples, mention may be made of: the dengue chimerivax™ products as described, for example, in patent application WO 98/37911, and the dengue/dengue chimeras as

described, for example, in patent applications WO 9640933 and WO0160847. The vaccinal dengue virus serotype 1 may, for example, be the VDV1 vaccinal strain or a ChimeriVax™ DEN-1, in particular a YF17D/DEN-1 virus, or else a 16007/PDK13 DEN-1 strain. The vaccinal dengue virus serotype 2 may, for 5 example, be the VDV2 vaccinal strain or a ChimeriVax™ DEN-2, in particular a YF17D/DEN-2 virus, or else a 16681/PDK53 DEN-2 strain. The vaccinal dengue virus serotype 3 may be a ChimeriVax™ DEN-3, in particular a YF17D/DEN-3 virus. The vaccinal dengue virus serotype 4 may be a ChimeriVax™ DEN-4, in particular a YF17D/DEN-4 virus. This strain was 10 described in patent application EP1159968 in the name of Mahidol University and was deposited with the Collection Nationale de Cultures de Microorganismes (CNCM) [National Collection of Microorganism Cultures] under the number I-2483.

“VDV” or “Vero dengue vaccine” denotes a live attenuated dengue viral 15 strain adapted on Vero cells and capable of inducing a specific humoral response, including the induction of neutralizing antibodies, in primates and in particular in humans.

“VDV-1” is a strain obtained from a wild-type strain DEN-1 16007 which was subjected to 11 passages on PDK cells (DEN-1 16007/PDK11), which was 20 then amplified on Vero cells at 32°C, and the RNA of which was purified and transfected into Vero cells. The VDV-1 strain has 14 additional mutations compared to the vaccinal strain DEN-1 16007/PDK13 (13 passages on PDK – Primary Dog Kidney – cells). The DEN-1 16007/PDK13 strain, also called “LAV1”, was described in patent application EP1159968 in the name of Mahidol 25 University and was deposited with the Collection Nationale de Cultures de Microorganismes (CNCM) under the number I-2480. The complete sequence of the VDV-1 strain is given in the sequence SEQ ID NO:1. Said strain can be readily reproduced from said sequence. A method of preparation and the characterization of the VDV-1 strain have been described in the International 30 patent application filed under the names of Sanofi-Pasteur and of the Center for Disease Control and Prevention under the number PCT/IB 2006/001313.

“VDV-2” is a strain obtained from a wild-type strain DEN-2 16681 which was subjected to 50 passages on PDK cells (DEN-2 16681/PDK50), and

plaque-purified, and the RNA of which was extracted and purified before being transfected into Vero cells. The VDV-2 strain was then obtained by plaque-purification and amplification on Vero cells. The VDV-2 strain has 10 additional mutations compared with the vaccinal strain DEN-2 16681/PDK53 (53 5 passages on PDK cells), 4 mutations of which are silent. The DEN-2 16681/PDK53 strain, also called "LAV2", was described in patent application EP1159968 in the name of Mahidol University and was deposited with the Collection Nationale de Cultures de Microorganismes (CNCM) under the number I-2481. The complete sequence of the VDV-2 strain is shown in the 10 sequence SEQ ID NO:2. The VDV-2 strain can be readily reproduced from said sequence. A method of preparation and of characterization of the VDV-2 strain has been described in the International patent application filed in the names of Sanofi-Pasteur and of the Center for Disease Control and Prevention under the number PCT/IB 2006/001513.

15 The VDV 1 and 2 strains are prepared by amplification on Vero cells. The viruses produced are harvested and clarified with respect to cell debris by filtration. The DNA is digested by enzymatic treatment. The impurities are removed by ultrafiltration. The infectious titers can be increased by means of a method of concentration. After the addition of a stabilizer, the strains are stored 20 in lyophilized or frozen form before use, and then reconstituted extemporaneously.

The term "ChimeriVax™ dengue" or "CYD" denotes a chimeric yellow fever (YF) virus which comprises the backbone of a YF virus in which the sequences encoding the premembrane and envelope proteins have been 25 replaced with those of a DEN virus. The term "CYD-1 or CYD DEN1" is thus used to describe a chimeric YF virus containing the prM and E sequences of a dengue serotype 1 strain (DEN-1). The term "CYD-2 or CYD DEN2" is used to describe a chimeric YF virus containing the prM and E sequences of a DEN-2 strain. The term "CYD-3 or CYD DEN3" is used to describe a chimeric YF virus 30 containing the prM and E sequences of a DEN-3 strain. The term "CYD-4 or CYD DEN4" is used to describe a chimeric YF virus containing the prM and E sequences of a DEN-4 strain. The preparation of these ChimeriVax™ dengues has been described in detail in International patent applications WO 98/37911

and WO 03/101397, to which reference may be made for a precise description of the method for preparing them. The chimeras described in the examples were generated using the prM and E sequences derived from the DEN1 PUO359 (TYP1140), DEN2 PU0218, DEN3 PaH881/88 and DEN4 1288 (TVP 5 980) strains. Any strain of the dengue virus could be used in the context of the present invention for the construction of the chimeras.

Preferably, the chimeric YF virus comprises the backbone of an attenuated yellow fever strain YF17D (Theiler M, and Smith HH (1937) J Exp. Med 65, p767-786.) (YF17D/DEN-1, YF17D/DEN-2, YF17D/DEN-3, 10 YF17D/DEN-4 virus). Examples of YF17D strains which can be used include YF17D204 (YF-Vax®, Sanofi-Pasteur, Swifwater, PA, USA; Stamaril®, Sanofi-Pasteur, Marcy l'Etoile, France; ARILVAX™, Chiron, Speke, Liverpool, UK; FLAVIMUN®, Berna Biotech, Bern, Switzerland); YF17D-204 France (X15067,X15062); YF17D-204,234 US (Rice et al., 1985, Science, 229:726- 15 733), or else related strains YF17DD (Genbank accession number U17066), YF17D-213 (Genbank accession number U17067) and the YF17DD strains described by Galler et al. (1998, Vaccines 16(9/10):1024-1028). Any other yellow fever virus strain attenuated for use in humans can be used in the context of the present invention for the construction of the chimeras.

20 A subject of the present invention is therefore also a bivalent composition or vaccine comprising an immunoeffective amount of a vaccinal dengue virus of a first serotype and of a vaccinal dengue virus of a second serotype and a pharmaceutically acceptable excipient.

25 For a description of the vaccinal viruses that can be used in the vaccines according to the invention, reference may be made to the description given thereof in the context of the method of immunization according to the invention.

According to a specific embodiment, the bivalent composition or vaccine according to the invention comprises CYD DEN-1 and CYD DEN-2, or CYD DEN-3 and CYD DEN-4, or CYD DEN-1 and CYD DEN-3 or CYD DEN-2 and 30 CYD DEN-4; advantageously, the vaccinal viruses are present in the vaccine in an amount of 10^5 CCID₅₀.

Each ChimeriVax™ monovalent vaccinal dengue virus (serotypes 1, 2, 3 and 4) was prepared by amplification of each serotype on Vero cells. More

specifically, the four viruses are produced separately on adherent Vero cells in serum-free medium. The viral harvest, clarified with respect to cell debris by filtration, is then concentrated and purified by ultrafiltration and chromatography in order to remove the host cell DNA. After the addition of a stabilizer, the 5 vaccinal strains are stored in frozen or lyophilized form before use, and are then reconstituted extemporaneously. The same method is applied for the four chimeras.

A dose, a composition or a vaccine is "monovalent" when it contains, in addition to a pharmaceutically acceptable excipient, a single dengue virus 10 serotype. A dose, a composition or a vaccine is "bivalent" when it contains two different dengue virus serotypes. A dose, a composition or a vaccine is "trivalent" when it contains three different dengue virus serotypes. A dose, a composition or a vaccine is "tetravalent" when it contains four different dengue virus serotypes. The multivalent compositions are obtained by simply mixing 15 the monovalent compositions.

The term "patient" denotes an individual (child or adult) who may be infected with dengue, in particular an individual at risk of infection, such as, for example, an individual who travels in regions where dengue is present or an inhabitant of these regions. This term therefore encompasses individuals who 20 are naive and also individuals who are non-naive with respect to the dengue virus.

Sequential immunization at separate anatomical sites

The inventors have shown in particular that the administration of the 4 25 serotypes in the form of two simultaneous bivalent administrations at separate anatomical sites, followed by a booster 30 days to 12 months after the first series of administrations, makes it possible to obtain an effective homologous protection against the 4 serotypes. The method according to the present invention is therefore most particularly valuable in the context of an 30 immunization strategy against dengue.

According to the present invention, the 4 dengue serotypes can be administered in any order provided that they are administered in pairs (i.e.

doses (i) and (ii), respectively, in the form of two monovalent doses or of a single bivalent dose) simultaneously at separate sites.

The method according to the present invention can therefore be implemented with the embodiments described below:

- 5 -(i) serotypes 1 and 2; (ii) serotypes 3 and 4; or
- (i) serotypes 1 and 3; (ii) serotypes 2 and 4; or
- (i) serotypes 1 and 4; (ii) serotypes 2 and 3; or
- (i) serotypes 2 and 3; (ii) serotypes 1 and 4; or
- (i) serotypes 2 and 4; (ii) serotypes 1 and 3; or
- 10 -(i) serotypes 3 and 4; (ii) serotypes 1 and 2.

Preferably, the method according to the present invention comprises the administration of the following vaccinal dengue viruses: (i) serotypes 1 and 2; (ii) serotypes 3 and 4 or (i) serotypes 1 and 3; (ii) serotypes 2 and 4. The doses (i) and (ii) are advantageously in the form of bivalent doses.

- 15 According to specific embodiments the present invention therefore covers the following schemes:

- (i) CYD DEN-1 and CYD DEN-2; (ii) CYD DEN-3 and CYD DEN-4
- (i) CYD DEN-1 and CYD DEN-3; (ii) CYD DEN-2 and CYD DEN-4
- (i) CYD DEN-1 and CYD DEN-4; (ii) CYD DEN-2 and CYD DEN-3
- 20 -(i) CYD DEN-2 and CYD DEN-3; (ii) CYD DEN-1 and CYD DEN-4
- (i) CYD DEN-2 and CYD DEN-4; (ii) CYD DEN-1 and CYD DEN-3
- (i) CYD DEN-3 and CYD DEN-4; (ii) CYD DEN-1 and CYD DEN-2
- (i) VDV-1 and CYD DEN-2; (ii) CYD DEN-3 and CYD DEN-4
- (i) VDV-1 and CYD DEN-3; (ii) CYD DEN-2 and CYD DEN-4
- 25 -(i) VDV-1 and CYD DEN-4; (ii) CYD DEN-2 and CYD DEN-3
- (i) CYD DEN-2 and CYD DEN-3; (ii) VDV-1 and CYD DEN-4
- (i) CYD DEN-2 and CYD DEN-4; (ii) VDV-1 and CYD DEN-3
- (i) CYD DEN-3 and CYD DEN-4; (ii) VDV-1 and CYD DEN-2
- (i) CYD DEN-1 and VDV-2 ; (ii) CYD DEN-3 and CYD DEN-4
- 30 -(i) CYD DEN-1 and CYD DEN-3; (ii) VDV-2 and CYD DEN-4
- (i) CYD DEN-1 and CYD DEN-4; (ii) VDV-2 and CYD DEN-3
- (i) VDV-2 and CYD DEN-3; (ii) CYD DEN-1 and CYD DEN-4
- (i) VDV-2 and CYD DEN-4; (ii) CYD DEN-1 and CYD DEN-3

- (i) CYD DEN-3 and CYD DEN-4; (ii) CYD DEN-1 and VDV-2
- (i) VDV-1 and VDV-2; (ii) CYD DEN-3 and CYD DEN-4
- (i) VDV-1 and CYD DEN-3; (ii) VDV-2 and CYD DEN-4
- (i) VDV-1 and CYD DEN-4; (ii) VDV-2 and CYD DEN-3
- 5 -(i) VDV-2 and CYD DEN-3; (ii) VDV-1 and CYD DEN-4
- (i) VDV-2 and CYD DEN-4; (ii) VDV-1 and CYD DEN-3, and
- (i) CYD DEN-3 and CYD DEN-4; (ii) VDV-1 and VDV-2.

Preferably, the method of immunization according to the invention comprises the administration of the following vaccinal dengue viruses: (i) CYD DEN-1 and CYD DEN 2; (ii) CYD DEN-3 and CYD DEN-4; or (i) CYD DEN-1 and CYD DEN-3; (ii) CYD DEN-2 and CYD DEN-4. The doses (i) and (ii) are advantageously in the form of bivalent doses.

The method of immunization according to the present invention comprises a second series of administrations implemented from 30 days to 12 months, advantageously from 30 days to 3 months, preferably 30 days, 45 days or 60 days, after the first series of administrations (i and ii), which advantageously comprises the administration of the same compositions as those used in the first series, which are advantageously administered under the same conditions.

20 In the context of the present invention, the term "dose of vaccinal virus" is intended to mean a composition comprising an "immunoeffective amount" of the vaccinal dengue virus, i.e. an amount of dengue virus sufficient to induce a homologous neutralizing antibody response, which can be demonstrated, for example, by means of the seroneutralization test as described below in 25 example 1. A serum is considered to be positive for the presence of neutralizing antibodies when the neutralizing antibody titer thus determined is greater than or equal to 1:10 (unit: 1/dilution).

Vaccinal strain amounts are commonly expressed in terms of viral 30 plaque-forming units (PFU) or of 50% tissue culture infectious dose, or else of 50% cell culture infectious dose (CCID₅₀). For example, the compositions according to the invention can contain from 10 to 10⁶ CCID₅₀, in particular from 10³ to 10⁵ CCID₅₀ of vaccinal dengue virus serotype 1, 2, 3 or 4 for a monovalent or bivalent composition. Thus, in the compositions or use

according to the invention, the doses of vaccinal dengue viruses serotypes 1, 2, 3 and 4 are preferably each within a range of from 10 to 10^6 CCID₅₀, such as 10, 10¹, 10², 10³, 10⁴, 10⁵ or 10⁶ CCID₅₀, in particular in a range from 10³ to 10⁵ CCID₅₀. The vaccinal viruses can be used at identical or different doses, which

5 can be adjusted according to the nature of the vaccinal virus used and to the strength of the immune response obtained.

According to a specific embodiment of the method according to the present invention, the monovalent or bivalent doses of vaccinal viruses comprise, respectively, 10⁵ CCID₅₀ of CYD DEN-1, of CYD DEN-2, of CYD 10 DEN-3 and of CYD DEN-4.

The neutralizing antibody response is advantageously a lasting response, i.e. it can be detected in the serum at least 6 months after the second series of administrations (i) and (ii).

15 The dose of a vaccinal dengue virus of a first serotype and the dose of a vaccinal dengue virus of a second serotype (i.e. dose(s) (i)) are administered simultaneously in the form of two monovalent compositions, or advantageously in the form of a single bivalent composition or dose.

Similarly, the dose of a vaccinal dengue virus of a third serotype and the dose of a vaccinal dengue virus of a fourth serotype (i.e. dose(s) (ii)) are 20 administered simultaneously in the form of two monovalent vaccinal compositions, or advantageously in the form of a single bivalent vaccinal composition.

The vaccinal viruses are administered in the form of vaccinal compositions or vaccinal virus doses which can be prepared according to any 25 method known to those skilled in the art. Usually, the viruses, generally in lyophilized form, are mixed with a pharmaceutically acceptable excipient, such as water or a phosphate buffered saline solution, wetting agents or stabilizers.

The term "pharmaceutically acceptable excipient" is intended to mean any solvent, dispersing medium, filler, etc., which does not produce a side reaction, 30 for example an allergic reaction, in humans or animals. The excipient is selected according to the pharmaceutical form chosen, and to the method and route of administration. Appropriate excipients and also the requirements in

terms of pharmaceutical formulation are described in "Remington: The Science & Practice of Pharmacy", which represents a reference work in the field.

Preferably, the vaccinal compositions are prepared in an injectable form, and can correspond to liquid solutions, suspensions or emulsions. The 5 compositions can in particular include an aqueous solution buffered so as to maintain a pH of between approximately 6 and 9 (as determined with a pH meter at ambient temperature).

Although it is not necessary to add an adjuvant, the compositions can nevertheless include such a compound, i.e. a substance which increases, 10 stimulates or strengthens the cellular or humoral immune response induced by the vaccinal strain administered simultaneously. Those skilled in the art are in a position to select, from the adjuvants conventionally used in the field of vaccines, an adjuvant which may be suitable in the context of the present invention.

15 The vaccinal compositions according to the invention can be administered according to any route normally used in immunization, for example parenterally (in particular intradermally, subcutaneously or intramuscularly), advantageously subcutaneously. Preferably, the vaccinal compositions are injectable compositions administered subcutaneously in the 20 left deltoid and the right deltoid region.

The volume of composition administered depends on the route of administration. For subcutaneous injections, the volume is generally between 0.1 and 1.0 ml, preferably approximately 0.5 ml.

25 The optimal period for the administration of all the serotypes 1 to 4, is approximately 1 to 3 months before exposure to the dengue virus. The vaccines can be administered as a prophylactic treatment for infection with a dengue virus in adults and children. Target populations therefore include individuals who may be naive (i.e. not previously immunized) or non-naive with respect to the dengue virus.

30 Vaccinal dengue virus serotypes 1 to 4 booster administrations can also be carried out, for example, between 6 months and 10 years, for example 6 months, 1 year, 3 years, 5 years or 10 years, after administration of the second series of administrations according to the invention. The booster

administrations will advantageously be implemented using the same vaccinal compositions (i.e. the same vaccinal viruses) and preferably under the same administration conditions (anatomical sites and routes of administration) as those used for the 1st and 2nd series of administrations.

5 The interference phenomena can be explained by the dominance of one or more serotypes compared with others, and are therefore independent of the technology used to manufacture the vaccine candidate (for example, VDV or chimerivax). The method according to the present invention can therefore apply in general to any vaccinal dengue virus.

10

A subject of the present invention is therefore also the use of doses of vaccinal dengue virus for the preparation of a vaccine for inducing a protection against the 4 dengue serotypes, comprising:

15 (a) a first series of administrations (i) of a dose of a vaccinal dengue virus of a first serotype and of a dose of a vaccinal dengue virus of a second serotype, and (ii) of a dose of a vaccinal dengue virus of a third serotype and of a dose of a vaccinal dengue virus of a fourth serotype, and

20 (b) a second series of administrations of doses (i) and (ii),
in which the doses (i) and (ii) are administered simultaneously at separate anatomical sites, and

in which the second series is implemented at least 30 days to at most 12 months after the first series.

25 For a description of the vaccinal dengue viruses that can be used in the context of the present invention, reference may be made to the description given thereof in relation to the method of immunization according to the invention.

30 A subject of the present invention is also a kit for immunization against the four dengue virus serotypes. The kit according to the present invention comprises the doses as defined above in relation to the method of immunization proposed. The kit according to the invention therefore comprises a case containing the various containers containing the vaccinal doses and

advantageously an instruction leaflet containing the information useful for administration of the vaccines.

According to one embodiment, the kit according to the invention comprises a case containing at least the vaccinal dengue viruses serotypes 1,

5 2, 3 and 4

(a) in the form of monovalent compositions contained in 4 separate containers, or

(b) in the form of two bivalent compositions contained in 2 separate containers.

10 According to another embodiment, the kit according to the invention comprises a case containing at least the vaccinal dengue viruses of a first serotype and of a second serotype,

(a) in the form of two monovalent compositions contained in 2 separate containers, or

15 (b) in the form of a bivalent composition contained in 1 single container.

For a description of the vaccinal dengue viruses that can be used in the kit according to the invention, reference may be made to the description of the vaccinal viruses given above in relation to the method of immunization
20 according to the invention.

According to a specific embodiment, the kit according to the present invention therefore comprises at least:

(a) a first container containing a bivalent vaccine comprising a ChimeriVax™ DEN-1 and a ChimeriVax™ DEN-2, and

25 (b) a second container containing a bivalent vaccine comprising a ChimeriVax™ DEN-3 and a ChimeriVax™ DEN-4.

According to another embodiment, the kit according to the invention comprises at least:

30 (a) a first container containing a bivalent vaccine comprising a ChimeriVax™ DEN-1 and a ChimeriVax™ DEN-3, and

(b) a second container containing a bivalent vaccine comprising a ChimeriVax™ DEN-2 and a ChimeriVax™ DEN-4.

According to another embodiment, the kit according to the invention comprises at least:

(a) a container containing a bivalent vaccine comprising a ChimeriVax™ DEN-1 and a ChimeriVax™ DEN-3, or

5 (b) a container containing a bivalent vaccine comprising a ChimeriVax™ DEN-2 and a ChimeriVax™ DEN-4, or

(c) a container containing a bivalent vaccine comprising a ChimeriVax™ DEN-1 and a ChimeriVax™ DEN-2, or

(d) a container containing a bivalent vaccine comprising a ChimeriVax™ DEN-3 and a ChimeriVax™ DEN-4.

The kits according to the invention may contain a single example or several examples of the containers as described above.

If the vaccines used are in lyophilized form, the kit will advantageously comprise at least one additional container containing the diluent for 15 reconstituting an injectable vaccinal dose. Any pharmaceutically acceptable diluent may be used to do this, conventionally water or a phosphate buffered aqueous solution.

The invention is illustrated by means of the following examples.

20

EXAMPLES

Example 1: Immunization in monkeys by simultaneous injection of two bivalent compositions at separate anatomical sites

The viremia and the immunogenicity were tested in a monkey model. 25 The viremia, in particular, was identified as one of the factors associated with the virulence and the severity of the disease in man and therefore constitutes an important parameter to be taken into consideration. The immunogenicity is, for its part, a key parameter in the context of the evaluation of the protection conferred.

30

1.1 Materials and methods:

The experiments in monkeys were carried out according to the European Directives relating to animal experimentation. The immunizations were carried

out in cynomolgus monkeys (*Macaca fascicularis*) originating from Mauritania. The monkeys were placed in quarantine for six weeks before immunization.

The monkeys were immunized subcutaneously in the arms with 0.5 ml of 5
vaccinal composition. After a light anesthesia with ketamine (Imalgene, Merial),
blood was collected by puncture from the inguinal or saphenous veins. At day 0
and 28 following each immunization, 5 ml of blood were sampled in order to
evaluate the antibody responses, while, between days 2 and 10, 1 ml of blood
was sampled in order to evaluate the viremia. The blood was collected on ice
and stored on ice until serum separation. To do this, the blood was centrifuged
10 for 20 minutes at 4°C and the serum collected was stored at -80°C until the
time of the tests.

Measurement of viremia

The post-vaccinal viremias were monitored by quantitative real-time 15
RP-PCR (qRT-PCR). Two sets of primers and of probes located in the NS5
gene of the DEN1 and DEN2 strains were used to quantify the VDV-1 RNA and
VDV-2 RNA, respectively. A third set of 2 primers and of 1 probe located in the
NS5 gene of the YF virus was used to quantify the CYD RNA. Finally, 4 sets of
primers and of probes specific for the various CYD serotypes, located at the
20 junction of the E (DEN)/NS1 (YF) genes were used to identify the serotype in
the samples positive for the YF NS5 RNA (see also table I). 7 plasmids
containing, under the control of the T7 promoter, the region targeted by each
PCR were transcribed *in vitro* so as to generate a series of synthetic RNAs
which were included as an internal reference in each RT-PCR assay. These
25 synthetic RNAs were assayed by spectrophotometry, and the amount of RNA
obtained was converted to number of RNA copies and expressed as GEQ
(genomic equivalents).

0.140 ml of monkey serum was extracted using the Macherey Nagel 30
"Nucleospin 96 virus™" RNA extraction kit, according to the manufacturer's
instructions, and then the purified RNA was eluted with 0.140 ml (0.090 ml,
then 0.05 ml) of RNase-free water. In order to avoid repeated freezing/thawing
cycles, a first quantification was carried out immediately after the extraction, on
5 µl of said RNA preparation. The remaining volume was frozen at 70°C.

The reaction mixtures contained, in addition to the components of the "Qiagen Qauntitect™ probes" RT-PCR quantification kit (Qiagen), 10 picomol of each primers, 4 picomol of each probe and 5 µl of RNA, in the total volume of 25 µl. In the case of the RNAs to be tested, 5 µl of the purified preparation 5 were directly introduced into the reaction mixture, without any prior dilution step. The synthetic RNAs were diluted to 1/10 in RNase-free water, and 7 dilutions containing approximately 10 to 10^6 GEQ in 5 µl were quantified in parallel in order to generate the standard curve.

The quantification reactions were carried out on the Applied Biosystem 10 ABI Prism 700™ device, using the following program: 50°C/30 min, 95°C/15 min, then 40 cycles of 95°C/15 sec–60°C/60 sec.

The limit of quantification of the viral RNA in this test is from 2.9 to 3.3 \log_{10} GEQ/ml (800 to 2000 GEQ/ml; 4 to 10 GEQ/reaction), according to the PCR targets (standard deviation: +/-0.3 \log_{10}).

15 The correlation between the infectious titer and the viral RNA quantification was established in parallel to the assays, by analysis of 0.140 ml of negative monkey serum samples (DO) to which a known amount of infectious particles of the viruses which were used for the immunization (CYD or VDV) were added. Said control sera were prepared at two dilutions 20 containing approximately 1 PFU and approximately 100 PFU in 5 µl (2.3 and 4.3 \log_{10} PFU/ml, respectively).

In the tests used in the examples, the correlation between GEQ and PFU is the following: GEQ/PFU ratio of 2.7 \log_{10} (i.e.: 1 PFU = 500 GEQ) for the sera positive for YF or CYDs. GEQ/PFU ratio of 2.5 \log_{10} (i.e.: 1 PFU = 320 25 GEQ) for the sera positive for VDV1 or VDV2.

The quantification limits being < 3.3 \log_{10} GEQ/ml (i.e.: < 4 PFU/ml) for qRT-PCR YF and CYDs and < 2.9 \log_{10} GEQ/ml (i.e.: < 2.5 PFU/ml) for qRT-PCR VDV1 and VDV2.

30 The primers and probes used are given in table 1 below, in which are listed, in order, for each assay, the sense and antisense primers and the probe.

Table 1

sequence		
YF	YF-NS5 sense	5' GCACGGATGTAACAGACTGAAGA (23 bases)
	YF NS5 anti	5' CCAGGCCGAACCTGTCAT (18 bases)
	YF-NS5	5' Fam- CGACTGTGTGGTCCGGCCATC-Tamra (22 bases)
CYD1 spe	CYD1- sense	5' CAT TGC AGT TGG CCT GGT AA (20 b)
	CYD1- anti	5' CTT TGG CAA GAG AGA GCT CAA GT (23 b)
	CYD1-	5' Fam-CCG ATC AAG GAT GCG CCA TCA-Tamra (21 b)
CYD2 spe	CYD2- sense	5' GTG GGA GTC GTG ACG CTG TA (20 b)
	CYD2- anti	5' GTT GAT GGC GCA TCC TTG ATC (21 b)
	CYD2	5' Fam-TGG GAG TTA TGG TGG GCG CCG-Tamra (21 b)
CYD3 spe	CYD3- sense	5' AAA ACA CTT CCA TGT CAT TTT CAT G (25b)
	CYD3- anti	5' GTT GAT GGC GCA TCC TTG ATC (21 b)
	CYD3-	5' Fam-TGCGATAGGAATTATCACACTCTATCTGGAGC-Tamra (33b)
CYD4 spe	CYD4- sense	5' CTT AGT ATT GTG GAT TGG CAC GAA (24 b)
	CYD4- anti	5' GCG CCA ACT GTG AAA CCT AGA (21 b)
	CYD4	5'-Fam-AGAAAACACTTCAATGGCAAATGACGTGCAT-Tamra (29 b)
VDV1 spe	VDV1-NS5 sense	5' TCG CAA CAG CCT TAA CAG C (19 b)
	VDV1-NS5 anti	5' ACT ATC TCC CTC CCA TCC TTC (21 b)
	VDV1-NS5	5' Fam-TTC ACA CCA CTT CCA C-MGB/NFQ (16 b)
VDV2 spec	VDV2-NS5 sense	5' AAT GAC AGA CAC GAC TCC (18 b)
	VDV2-NS5 anti	5' CCC AAA ACC TAC TAT CTT CAA C (22 b)
	VDV2-NS5	5' Fam-TGG AAG TCG GCA CGT GA-MGB/NFQ (17 b)

Measurement of neutralizing antibodies (seroneutralization test) (SN50)

5 Conventionally, the dengue antibody measurement is established using the PRNT50 (50% PFU number reduction neutralization test). Since this test is laborious and uses up a lot of material, we developed the SN50 test, based on 50% reduction in the number of units measured in a CCID50 test.

In a 96-well plate, 0.120 ml of each decomplemented serum is added to 10 0.480 ml of diluent (ISCOVE 4% FCS) per well. 6-fold serial dilutions are prepared by transfer of 0.150 ml of serum into 0.450 ml of diluent. 450 μ l of virtual dilution at $2.7 \log_{10}$ CCID50/ml are added to each well so as to obtain 25 CCID50/well. The plate is incubated at 37°C for 1 hour. 0.1 ml of each dilution is then distributed into 6 wells of a 96-well plate into which VERO cells 15 had been seeded 3 days before the beginning of the experiment at a density of 8000 cells/well, in 0.1 ml of ISCOVE medium containing 4% FCS. After incubation at 37°C for 6 days, in the presence of 5% CO₂, the cells are fixed with an ethanol/acetone (70/30) mixture at 4°C for 15 minutes, and then washed 3 times in PBS and incubated for 1 h at 37°C in the presence of 20 0.05 ml of a 1/2000 dilution of an anti-flavivirus monoclonal antibody (mAb

4G2). The plates are then washed twice and incubated for 1 h at 37°C in the presence of 0.05 ml of a 1/1000 dilution of an alkaline phosphatase-conjugated anti-mouse IgG. The lysis plaques are visualized by adding 0.05 ml of a colored substrate: BCIP/NBT. The neutralizing antibody titers are calculated using the

5 Karber formula as defined below:

$$\log_{10}SN50 = d + f/N (X + N/2),$$

in which:

10 d represents the dilution resulting in 100% neutralization (i.e. 6 negative replicates, i.e. replicates exhibiting no sign of infection)
f: represents the dilution factor in log10 (e.g. dilution factor of 1:4, f = 0.6)
N: represents the number of replicates/dilution (N=6)
X: total number of wells exhibiting no sign of infection, with the exception of the
15 dilution d

The limit of viral detection is 10 SN50 (i.e. 1.0 log₁₀SN50).

The viral strains which were used for the neutralization are the DEN1 16007, DEN2 16681, DEN3 16562 or DEN4 1036 strains.

For the controls, the initial viral dilutions were re-titrated.

20 The correlation between the neutralizing titer measured in the SN50 test and the neutralizing titer measured conventionally in the PRNT50 test is:
$$\log_{10}PRNT50 = \log_{10}SN50 + 0.2$$

25 The mean titer (GMT) is established by calculating the geometric mean of the titers expressed as a linear value, samples of which the titer is less than the detection threshold being assigned, by convention, a value equal to half this threshold.

1.2 Evaluation of the sequential immunizations

30 2 groups of 4 monkeys of equivalent age and weight were immunized (see table 2).

The immunization was carried out subcutaneously in the arm, with a 23G1 needle, at a dose of 10^5 CCID₅₀ for each serotype for the CYD DEN 1 to 4 vaccines.

Table 2: Composition of the groups and immunization protocol

5

Monkeys		
Group	Immunizations	
	D0	D58
1	CYD-1,2 in one arm CYD-3,4 in the other arm	CYD-1,2 in one arm CYD-3,4 in the other arm
2	CYD-1,2,3,4	CYD-1,2,3,4

The immunogenicity results obtained after one immunization (D28) and two immunizations (D86) are given in table 3.

The viremia results are given in table 4.

Table 3: SN50 neutralizing titer (units 1/dil)

Group	ID	Monkeys		D0+28				D58+28			
		D0	Immunizations D58	DEN-1	DEN-2	DEN-3	DEN-4	DEN-1	DEN-2	DEN-3	DEN-4
1	AP545	CYD 1,2 in one arm	CYD 1,2 in one arm	20	25	-	50	40	50	-	319
	AO949	CYD 3,4 in the other arm	CYD 3,4 in the other arm	20	-	-	20	319	20	13	100
	AP335			20	-	-	252	100	16	10	200
	AP817			20	16	32	40	319	25	32	402
	geometric mean			20	10	8	56	142	25	12	225
	AP676			63	-	-	126	100	-	-	40
2	AQ005			25	-	-	63	50	-	-	63
	AP961	CYD 1234	CYD 1234	50	-	-	158	80	-	80	400
	AQ163			63	-	-	40	100	-	16	252
	geometric mean			47	<10	<10	84	80	<10	13	126

-: titer < 10

Table 4: Viremia analysis (units: log10 GEQ/ml)

Group	Monkey	Primary immunization							Booster							
		D2	D3	D4	D6	D7	D8	D9	D10	D58	D59	D62	D63	D64	D65	D66
1 CYD1,2 + CYD3,4 2 points of injection	AP545	3.17	-	-	-	3.12	3.67	4.55	4.59	-	-	-	-	-	-	-
	AO949	3.93	3.66	3.34	4.57	-	-	-	-	-	-	-	-	-	-	-
	AP335	3.36	3.06	3.34	3.83	4.05	4.41	4.24	3.64	-	-	-	-	-	-	-
	AP817	4.17	3.99	3.61	-	-	-	-	2.89	-	-	-	-	-	-	-
2 CYD 1,2,3,4 1 point of injection	AP676	-	-	-	-	-	-	3.65	-	-	-	-	-	-	-	-
	AQ005	3.19	-	3.35	-	-	-	-	3.41	-	-	-	-	-	-	-
	AP961	-	-	-	-	-	3.86	3.42	3.29	3.56	-	-	-	-	-	-
	AQ163	3.18	3.16	-	3.30	3.60	2.95	3.00	-	-	-	-	-	-	-	-

Serotypes

CYD1	
CYD2	
CYD3	
CYD4	
CYD1+4	

Briefly, the results can be summarized as follows:

- The administration scheme according to the present invention makes it possible to qualitatively and quantitatively increase the homologous neutralizing antibody response which is obtained with the tetravalent immunization.

- The bivalent immunization CYD-1,2 concomitant with a CYD-3,4 immunization carried out at a separate anatomical site induces, after booster, homologous responses against the four serotypes in all the monkeys, except for serotype 3 in one animal.

- Furthermore, the responses against serotypes 1 and 4 have a tendency to be higher in the case of simultaneous bivalent immunizations than with tetravalent immunization at a single site.

- The viremia (table 4) is predominantly caused by CYD-4 whether this is after simultaneous bivalent administration or tetravalent administration. It can therefore be concluded therefrom that separation of the serotypes does not promote the emergence of a serotype 1, 2 and 3 viremia.

The examples therefore show that the method of immunization according to the present invention improves the immunogenicity of the vaccinal dengue viruses without impairing the safety of the latter.

Example 2 Immunization by simultaneous injection of two bivalent compositions CYD-1,4 and CYD-2,3 at distinct anatomical sites in monkeys

The viremia and the immunogenicity were tested in the monkey model as in example 1. In the present example, the bivalent compositions tested contain, respectively, the most immunogenic vaccinal viruses (CYD-1,4) and the least immunogenic vaccinal viruses (CYD-2,3).

2.1 Materials and methods: Identical to example 1

2.2 Evaluation of the simultaneous immunizations

Two groups of 4 monkeys of equivalent age and weight were immunized (see table 5).

The immunization was carried out as described in example 1.

Table 5: Composition of the groups and immunization protocol

Group	Monkeys	
	Immunizations	
	D0	D58
1	CYD 1,4 in one arm CYD 2,3 in the other arm	CYD 1,4 in one arm CYD 2,3 in the other arm
2	CYD 1234	CYD 1234

The immunogenicity results obtained after one immunization (D28) and two immunizations (D86) are given in table 6.

The viremia results are similar to those obtained in example 1, showing viremia induced by serotype 4 and no significant differences between the two groups.

Table 6: SN50 neutralizing titer (units 1/dil)

		Monkeys				D0+28				D58+28			
Group	ID	Immunizations		DEN-1	DEN-2	DEN-3	DEN-4	DEN-1	DEN-2	DEN-3	DEN-4	DEN-1	DEN-2
		D0	D58	63	10	<	100	200	25	1	2	3	10
1	AR465			13	<	<	126	401	<	20	20	20	126
	AR558			<	<	<	100	13	<	<	<	<	160
	AR559	CYD 1234	CYD 1234	25	<	<	318	201	<	<	<	<	50
	AR639			20	<	<	142	119	<	<	<	<	201
	Geometric mean												119
2	AR083	CYD 1,4 in one arm	CYD 1,4 in one arm	63	<	<	201	100	16	10	126		
	AR506	CYD 2,3 in the other arm	CYD 2,3 in the other arm	63	25	13	638	126	100	32	201		
	AR610			63	20	<	100	159	50	40	253		
	AR644			40	<	<	40	505	80	40	100		
	Geometric mean			56	<	<	150	178	50	27	159		

<: titer < 10

The results support those obtained in example 1 and can be summarized as follows:

- The administration scheme makes it possible to qualitatively and quantitatively increase the homologous neutralizing antibody response which is obtained with the tetravalent immunization.

- The bivalent immunization CYD-1,4 concomitant with a CYD-2,3 immunization carried out at a separate anatomical site induces, after booster, homologous responses against the four serotypes in all the monkeys, which is not the case in the conventional tetravalent group, as seen in example 1.

- Compared with those of the group of monkeys having received two bivalents CYD-1,2 and CYD-3,4 in example 1, the antibody titers observed after bivalent immunization CYD-1,4 concomitant with a CYD-2,3 immunization are higher for serotypes 1, 2 and 3, and lower for serotype 4, which shows a more balanced response between the 4 serotypes, less dominated by serotype 4.

- Separating the dominant serotypes from the others in such an immunization scheme enabled a balanced response against the 4 serotypes.

The 2 examples above therefore show that the immunization method according to the present invention improves the immunogenicity of the vaccinal dengue viruses without impairing the safety of the latter, as evaluated by measuring the viremia.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

The reference in this specification to any prior publication (or information derived from it), or to any matter which is known, is not, and should not be taken as an acknowledgment or admission or any form of suggestion that that prior publication (or information derived from it) or known matter forms part of the common general knowledge in the field of endeavour to which this specification relates.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method for inducing a protection against the 4 dengue serotypes in a patient, comprising:

(a) a first series of administrations (i) of a dose of a vaccinal dengue virus of a first serotype and of a dose of a vaccinal dengue virus of a second serotype, and (ii) of a dose of a vaccinal dengue virus of a third serotype and of a dose of a vaccinal dengue virus of a fourth serotype, and

(b) a second series of administrations of doses (i) and (ii), in which the doses (i) and (ii) are administered simultaneously at separate anatomical sites, and

in which the second series is implemented at least 30 days to at most 12 months after the first series, and

in which said vaccinal dengue viruses of a first, a second, a third and a fourth serotype are respectively:

vaccinal dengue viruses of serotypes 1, 2, 3 and 4;

vaccinal dengue viruses of serotypes 1, 3, 2 and 4;

vaccinal dengue viruses of serotypes 1, 4, 2 and 3;

vaccinal dengue viruses of serotypes 2, 3, 1 and 4;

vaccinal dengue viruses of serotypes 2, 4, 1 and 3; or

vaccinal dengue viruses of serotypes 3, 4, 1 and 2.

2. The method as claimed in claim 1, in which the vaccinal dengue viruses (i) are administered in the form of a single bivalent dose.

3. The method as claimed in claim 1 or 2, in which the vaccinal dengue viruses (ii) are administered in the form of a single bivalent vaccinal dose.

4. The method as claimed in any one of claims 1 to 3, in which said vaccinal dengue virus of serotype 1 is selected from the group consisting of the VDV1 strain and of a ChimeriVax™ DEN-1.

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5. The method as claimed in any one of claims 1 to 4, in which said vaccinal dengue virus of serotype 2 is selected from the group consisting of the VDV2 strain and of a ChimeriVax™ DEN-2.

6. The method as claimed in any one of claims 1 to 5, in which said vaccinal dengue virus of serotype 1 is the VDV1 strain and said vaccinal dengue virus serotype 2 is the VDV2 strain.

7. The method as claimed in any one of claims 1 to 5, in which said vaccinal dengue virus of serotype 1 is a ChimeriVax™ DEN-1 and said vaccinal dengue virus serotype 2 is a ChimeriVax™ DEN-2.

8. The method as claimed in any one of claims 1 to 7, in which said vaccinal dengue virus of serotype 3 is a ChimeriVax™ DEN-3.

9. The method as claimed in any one of claims 1 to 8, in which said vaccinal dengue virus of serotype 4 is a ChimeriVax™ DEN-4.

10. The method as claimed in any one of claims 1 to 9, in which the first and second serotypes are, respectively, serotype 1 and serotype 2 and the third and fourth serotypes are, respectively, serotype 3 and serotype 4.

11. The method as claimed in any one of claims 1 to 9, in which the first and second serotypes are, respectively, serotype 1 and serotype 4 and the third and fourth serotypes are, respectively, serotype 2 and serotype 3.

12. The method as claimed in any one of claims 1 to 5 and 7 to 9, in which the first and second serotypes are, respectively, CYD DEN-1 and CYD DEN-2 and the third and fourth serotypes are, respectively, CYD DEN-3 and CYD DEN-4 .

13. The method as claimed in any one of claims 1 to 5 and 7 to 9, in which the first and second serotypes are, respectively, CYD DEN-1 and CYD DEN-4 and the third and fourth serotypes are, respectively, CYD DEN-2 and CYD DEN-3.

14. The method as claimed in any one of claims 1 to 13, in which the amount of dengue vaccinal viruses of serotypes 1, 2, 3 and 4 is within a range of from 10^3 to 10^6 CCID₅₀.

15. The method as claimed in any one of claims 1 to 14, in which the vaccinal viruses used in the second series of administrations are identical to those used in the first series of administrations.

16. The method as claimed in any one of claims 1 to 14, in which the second series of administrations is implemented 30 days to 60 days after the first series of administrations.

17. A kit when used for immunization against the dengue virus, comprising a case containing at least the vaccinal dengue viruses of serotypes 1, 2, 3 and 4

(a) in the form of monovalent compositions contained in 4 separate containers, or

(b) in the form of two bivalent compositions contained in 2 separate containers;

wherein said monovalent compositions or said bivalent compositions are used in a method for inducing a protection against the 4 dengue serotypes 1-4 in a patient, comprising:

a first series of administrations (i) of a dose of a vaccinal dengue virus of a first serotype and of a dose of a vaccinal dengue virus of a second serotype, and (ii) of a dose of a vaccinal dengue virus of a third serotype and of a dose of a vaccinal dengue virus of a fourth serotype; and

a second series of administrations of doses (i) and (ii), in which the doses (i) and (ii) are administered simultaneously at separate anatomical sites, and

in which the second series is implemented at least 30 days to at most 12 months after the first series, and

in which said vaccinal dengue viruses of a first, a second, a third and a fourth serotype are respectively:

vaccinal dengue viruses of serotypes 1, 2, 3 and 4;

vaccinal dengue viruses of serotypes 1, 3, 2 and 4;

vaccinal dengue viruses of serotypes 1, 4, 2 and 3;

vaccinal dengue viruses of serotypes 2, 3, 1 and 4;

vaccinal dengue viruses of serotypes 2, 4, 1 and 3; or

vaccinal dengue viruses of serotypes 3, 4, 1 and 2.

18. The kit as claimed in claim 17, comprising at least:

(a) a first container containing a bivalent vaccine comprising a ChimeriVax™ DEN-1 and a ChimeriVax™ DEN-2, and

(b) a second container containing a bivalent vaccine comprising a ChimeriVax™ DEN-3 and a ChimeriVax™ DEN-4.

19. The kit as claimed in claim 18, comprising at least:

(a) a first container containing a bivalent vaccine comprising a ChimeriVax™ DEN-1 and a ChimeriVax™ DEN-3, and

(b) a second container containing a bivalent vaccine comprising a ChimeriVax™ DEN-2 and a ChimeriVax™ DEN-4.

20. The use of doses of vaccinal dengue virus for the preparation of a vaccine for inducing a protection against the 4 dengue serotypes 1-4, comprising:

(a) a first series of administrations (i) of a dose of a vaccinal dengue virus of a first serotype and of a dose of a vaccinal dengue virus of a second serotype, and (ii) of a dose of a vaccinal dengue virus of a third serotype and of a dose of a vaccinal dengue virus of a fourth serotype, and

(b) a second series of administrations of doses (i) and (ii),

in which the doses (i) and (ii) are administered simultaneously at separate anatomical sites, and

in which the second series is implemented at least 30 days to at most 12 months after the first series, and

in which said vaccinal dengue viruses of a first, a second, a third and a fourth serotype are respectively:

vaccinal dengue viruses of serotypes 1, 2, 3 and 4;

vaccinal dengue viruses of serotypes 1, 3, 2 and 4;

vaccinal dengue viruses of serotypes 1, 4, 2 and 3;

vaccinal dengue viruses of serotypes 2, 3, 1 and 4;

vaccinal dengue viruses of serotypes 2, 4, 1 and 3; or

vaccinal dengue viruses of serotypes 3, 4, 1 and 2.

21. The method as claimed in claim 1, or a kit as claimed in claim 17, substantially as herein described.

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