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(54) **Title:** THERMOREVERSIBLE OIL-IN-WATER EMULSION

(54) **Titre :** EMULSION HUILE DANS EAU THERMOREVERSIBLE

(57) **Abstract:** The invention relates to a thermoreversible oil-in-water emulsion which comprises: a TLR4 agonist whose chemical structure does not include a sugar ring, squalene, a nonionic surfactant belonging to the polyoxyethylene alkyl ether chemical group, a hydrophilic surfactant and an aqueous solvent, and which exhibits immunostimulating properties.

(57) **Abrégé :** L'invention se rapporte à une emulsion huile dans eau, thermoréversible, comprenant : un agoniste du TLR4 dont la structure chimique ne comporte pas de noyau sucré, du squalène, un tensioactif non ionique appartenant au groupe chimique des polyoxyethylène alkyl éther, un tensioactif hydrophile, un solvant aqueux, et qui manifeste des propriétés immunostimulantes.

WO 2007/080308 A2

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2007204295 22 Oct 2012

THERMOREVERSIBLE OIL-IN-WATER EMULSION

The invention relates to an immunostimulating composition in the form of a thermoreversible oil-in-water (O/W) emulsion comprising a TLR4 agonist, called TLA4.

TLR4 (toll-like receptor type 4) is a receptor expressed by antigen-presenting cells of the immune system; it is involved in early defense mechanisms against gram- bacterial infections. The lipopolysaccharide (LPS) of gram- bacteria is the natural ligand for TLR4; it activates the receptor, which triggers a cascade of biochemical events, in particular the activation of Nf-Kappa B transcription factor, and the production of pro-inflammatory cytokines. Monophosphoryl lipid A, which comes from the hydrolysis of LPS, is also a ligand for TLR4, with the advantage that it is less toxic than LPS.

WO 2004/060396 describes formulations in the form of O/W emulsions containing a phospholipid adjuvant. The emulsions, which have a submicronic size, are obtained by means of a high pressure homogenizer (microfluidizer). The method of production uses high mechanical energies in order to obtain sufficiently great shear forces to reduce the size of the oil drops. According to this teaching, the emulsion obtained contains drops whose size is approximately 500 nm.

It is desirable to be able to have an alternative formulation to that proposed in that patent application, and especially one which can be obtained by means of a simpler method (not requiring specific shear technology), involving low energy while at the same time being reproducible, reliable and usable on a large scale; furthermore, the adjuvant formulation must be able to improve the effectiveness of vaccines, by increasing the immune response to an antigen, while at the same time not exhibiting any sign of toxicity which would be detrimental to the completely safe administration thereof.

To this effect, a subject of the invention is:

An Oil-in-Water (O/W) emulsion comprising:

i) a TLR4 agonist, called TLA4, in which TLA4 is a chemical compound of formula I, II, III or IV,

2007204295 22 Oct 2012

ii) squalene,

iii) an aqueous solvent,

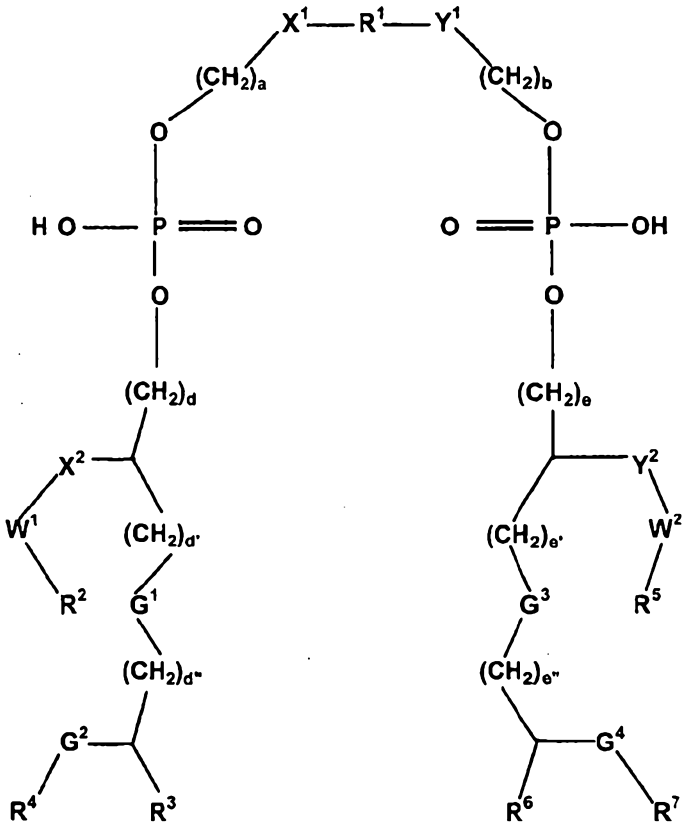
iv) a nonionic hydrophilic surfactant which is a polyoxyethylene alkyl ether,

v) a nonionic hydrophobic surfactant, and

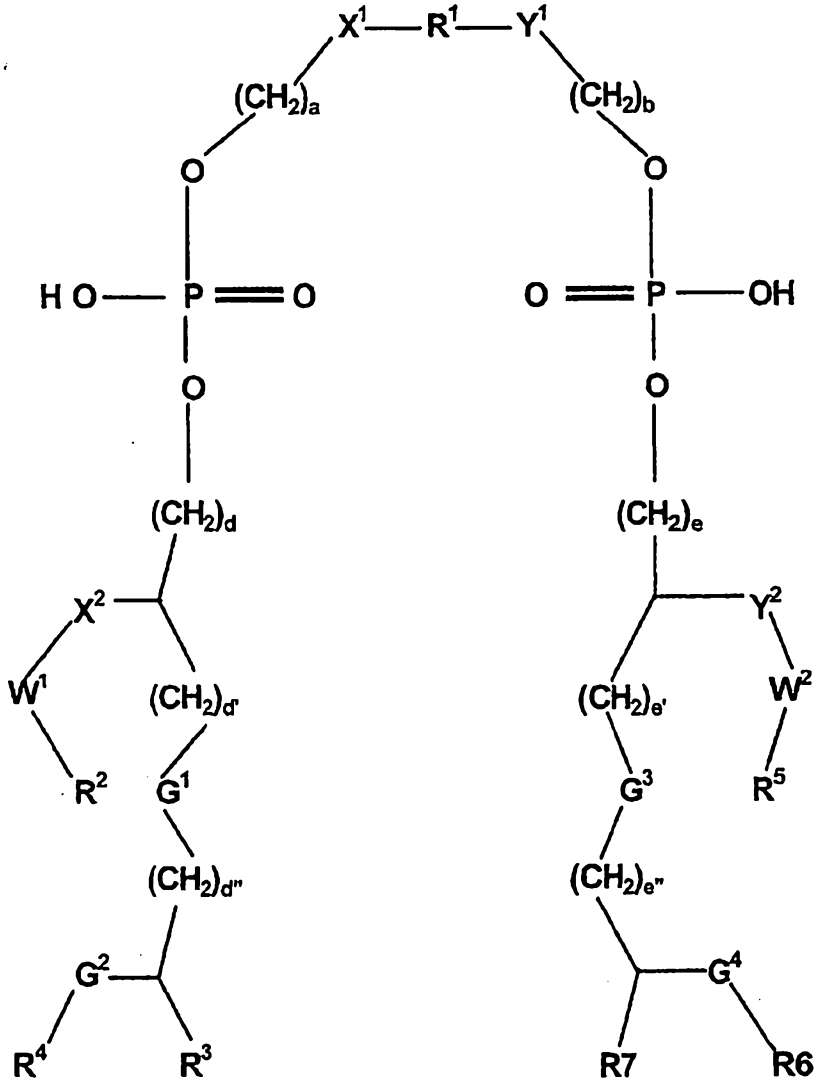
which is thermoreversible;

wherein formula I, II, III and IV are as follows:

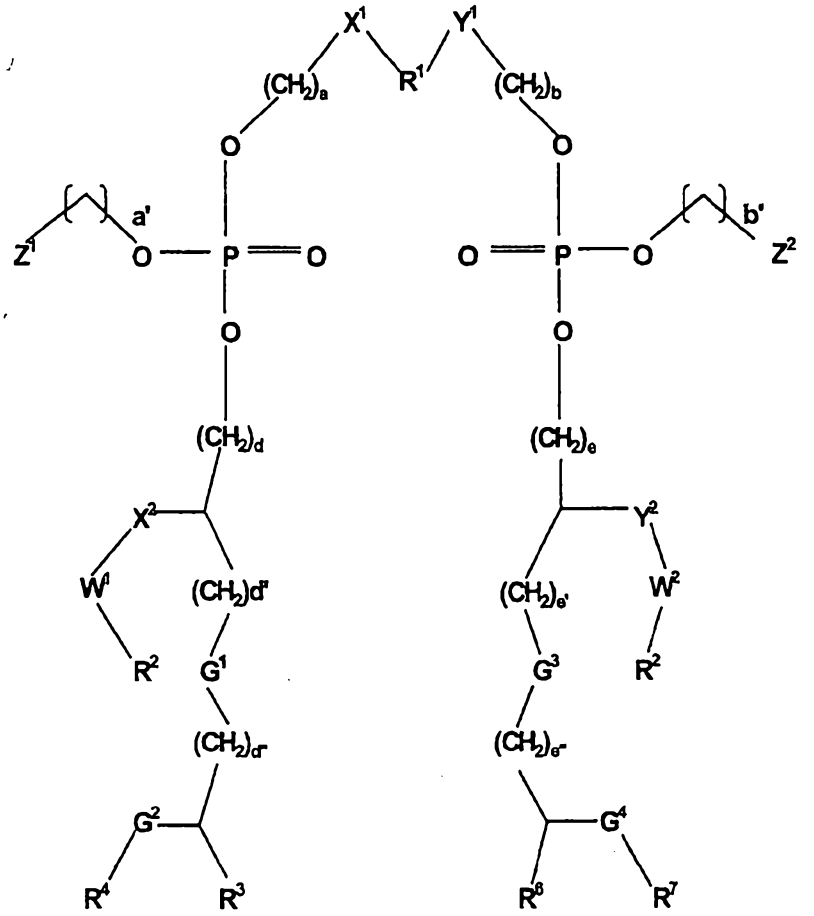
Compound of formula I



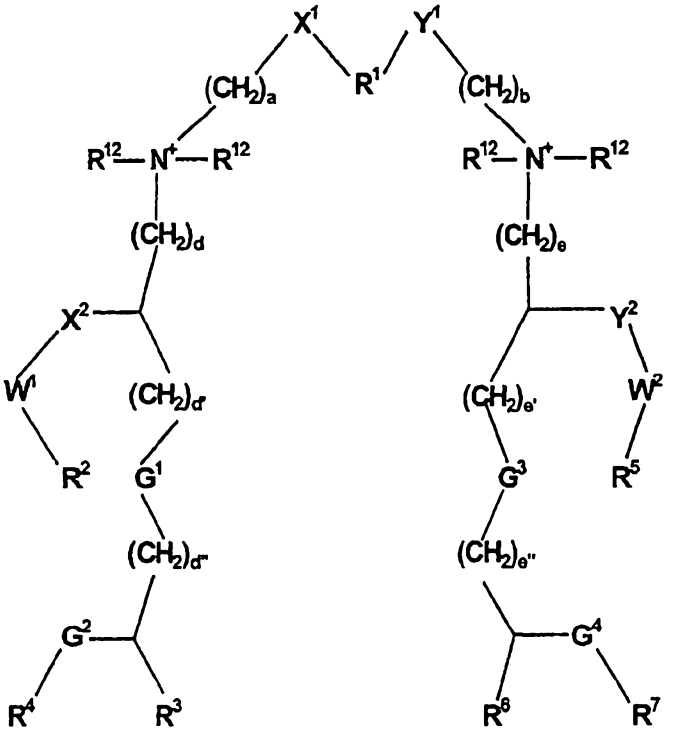
Compound of formula II



Compound of formula III



Compound of formula IV



in which, for each of formula I, II, III or IV, R^1 is selected from the group consisting of:

a) C(O);

b) C(O)-(C_1-C_{14} alkyl)-C(O), in which said C_1-C_{14} alkyl is optionally substituted with a hydroxyl, a C_1-C_5 alkoxy, a C_1-C_5 alkylenedioxy, a (C_1-C_5 alkyl)amino or a (C_1-C_5 alkyl)aryl, in which said aryl moiety of said (C_1-C_5 alkyl)aryl is optionally substituted with a C_1-C_5 alkoxy, a (C_1-C_5 alkyl)amino, a (C_1-C_5 alkoxy)amino, a (C_1-C_5 alkyl)amino(C_1-C_5 alkoxy), -O-(C_1-C_5 alkyl)amino (C_1-C_5 alkoxy), -O-(C_1-C_5 alkyl)amino-C(O)-(C_1-C_5 alkyl)-C(O)OH, or -O-(C_1-C_5 alkyl)amino-C(O)-(C_1-C_5 alkyl)-C(O)-(C_1-C_5)alkyl;

c) an alkyl comprising a C_2-C_{15} linear or branched chain, optionally substituted with a hydroxyl or an alkoxy; and

d) -C(O)-(C_6-C_{12} arylene)-C(O)- in which said arylene is optionally substituted with a hydroxyl, a halogen, a nitro or an amino;

a and b are independently 0, 1, 2, 3 or 4;

d, d', d", e, e' and e" are independently 0, 1, 2, 3 or 4;

X^1, X^2, Y^1 and Y^2 are independently selected from the group consisting of null, an oxygen, NH and N (C(O)(C_1-C_4 alkyl)), and N(C_1-C_4 alkyl);

W^1 and W^2 are independently selected from the group consisting of a carbonyl, a methylene, a sulfone and a sulfoxide;

R^2 and R^5 are independently selected from the group consisting of:

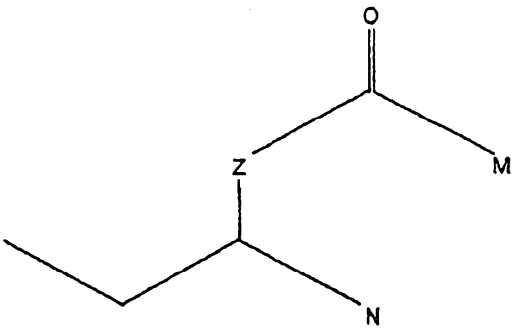
a) a C_2 to C_{20} straight chain or branched chain alkyl, which is optionally substituted with an oxo, a hydroxyl or an alkoxy;

b) a C_2 to C_{20} straight chain or branched chain alkenyl or dialkenyl, which is optionally substituted with an oxo, a hydroxyl or an alkoxy;

c) a C_2 to C_{20} straight chain or branched chain alkoxy, which is optionally substituted with an oxo, a hydroxyl or an alkoxy;

d) NH-(C_2 to C_{20} straight chain or branched chain alkyl), in which said alkyl group is optionally substituted with an oxo, a hydroxy or an alkoxy; and

e)



in which Z is selected from the group consisting of an O and NH, and M and N are independently selected from the group consisting of an alkyl, an alkenyl, an alkoxy, an acyloxy, an alkylamino and an acylamino comprising a C_2-C_{20} linear or branched chain;

R^3 and R^6 are independently selected from the group consisting of a C_2 to C_{20} straight chain or branched chain alkyl or alkenyl, optionally substituted with an oxo or a fluoro;

R^4 and R^7 are independently selected from the group consisting of a C(O)-(C_2 to C_{20} straight chain or branched chain alkyl or alkenyl), a C_2 to C_{20} straight chain or branched chain alkyl, a C_2 to C_{20} straight chain or branched chain alkoxy, and a C_2 to C_{20} straight chain or branched chain alkenyl; in which said alkyl, alkenyl or alkoxy groups can be independently and optionally substituted with a hydroxyl, a fluoro or a C_1-C_5 alkoxy;

G_1, G_2, G_3 and G_4 are independently selected from the group consisting of an oxygen, a methylene, an amino, a thiol, -C(O)NH-, -NHC(O)-, and -N(C(O)(C_1-C_4 alkyl))-;

or G^2R^4 or G^4R^7 can together be a hydrogen atom or a hydroxyl;

and in which, for formula III:
a' and b' are independently 2, 3, 4, 5, 6, 7 or 8, preferably 2;
Z^1 is selected from the group consisting of $-OP(O)(OH)_2$, $-P(O)(OH)_2$, $-OP(O)(OR^8)(OH)$ where R^8 is a C_1-C_4 alkyl chain, $-OS(O)_2OH$, $-S(O)_2OH$, $-CO_2H$, $-OB(OH)_2$, -OH, $-CH_3$, $-NH_2$ and $-NR^9_3$ where R^9 is a C_1-C_4 alkyl chain;

Z^2 is selected from the group consisting of $-OP(O)(OH)_2$, $-P(O)(OH)_2$, $-OP(O)(OR^{10})(OH)$ where R^{10} is a C_1-C_4 alkyl chain, $-OS(O)_2OH$, $-S(O)_2OH$, $-CO_2H$, $-OB(OH)_2$, -OH, $-CH_3$, $-NH_2$ and $-NR^{11}$ where R^{11} is a C_1-C_4 alkyl chain;

and in which, for formula IV:
R12 is H or a C_1-C_4 alkyl chain;
or a pharmaceutically acceptable salt of the compound of formula I, II, III or IV.

The emulsion according to the invention is thermoreversible, which means that it changes from the state of an O/W emulsion to the state of a W/O emulsion when it is heated to a temperature at least equal to a "phase inversion temperature". On the microscopic scale, the phase inversion temperature reflects the change from a curvature directed toward the oily phase to a curvature directed toward the aqueous phase, this

transition necessarily involving the passing through a zero mean curvature phase (the system then being related either to a lamellar phase or to a microemulsion).

The emulsion according to the invention can be obtained by a temperature-variation phase inversion process, which is highly advantageous from an industrial point of view since it can be readily controlled and adapted to large production volumes. Such a process guarantees safety and cost-effectiveness, both of which are necessary for the pharmaceutical industry. In addition, by virtue of this process, it is possible to obtain a monodisperse emulsion in which the droplet size is small, which makes the emulsion readily filterable by means of sterilizing filters for which the cut-off is 200 nm.

Advantageously, at least 90% of the population by volume of the oil droplets of the emulsion according to the invention has a size $\leq$ 200 nm. In general, at least 50% of the population by volume of the oil droplets of these emulsions has a size $\leq$ 110 nm. According to one specific characteristic, at least 90% of the population by volume of the oil droplets has a size $\leq$ 180 nm and at least 50% of the population by volume of the droplets has a size $\leq$ 110 nm.

In general, the thermoreversible emulsion according to the invention is homogeneous. The term "homogeneous emulsion" is intended to mean an emulsion for which the graphic representation of size distribution ("granulogram") of the oil droplets is unimodal. Typically, this graphic representation is of the "Gaussian" type.

The size of the droplets can be measured by various means, and in particular using laser diffraction particle size analyzers such as the Beckman Coulter devices of the LS range (in particular the LS230) or Malvern devices of the Mastersizer range (in particular the Mastersizer 2000). The principle of measurement of these devices is based on analyzing the intensity of the light scattered by the particles as a function of the angle (large, medium and small angle detectors) when the sample is illuminated by a laser beam. This analysis is carried out by means of mathematical models chosen according to the size and the nature of the material used. In the case of the measurement of the size of submicronic particles, it is necessary to apply a specific optical model (Mie theory)

taking into account the refractive indices of the oily phase (in this case, 1.495 for squalene) and of the aqueous phase (for example, it is 1.332 for water); it is also necessary to be able to detect the weak intensities emitted by the very fine particles, which requires optimizing the analysis with:

- an additional detection cell for the large-angle polarized intensity differential scattering measurement (PIDS system from Coulter, which allows measurement from 40 nm),

- a detection system combining 2 wavelengths, blue and red light, from Malvern. The source of shorter-wavelength blue light, associated with wide-angle scattering and backscatter detectors reinforces the performance levels of the analysis of the submicronic range.

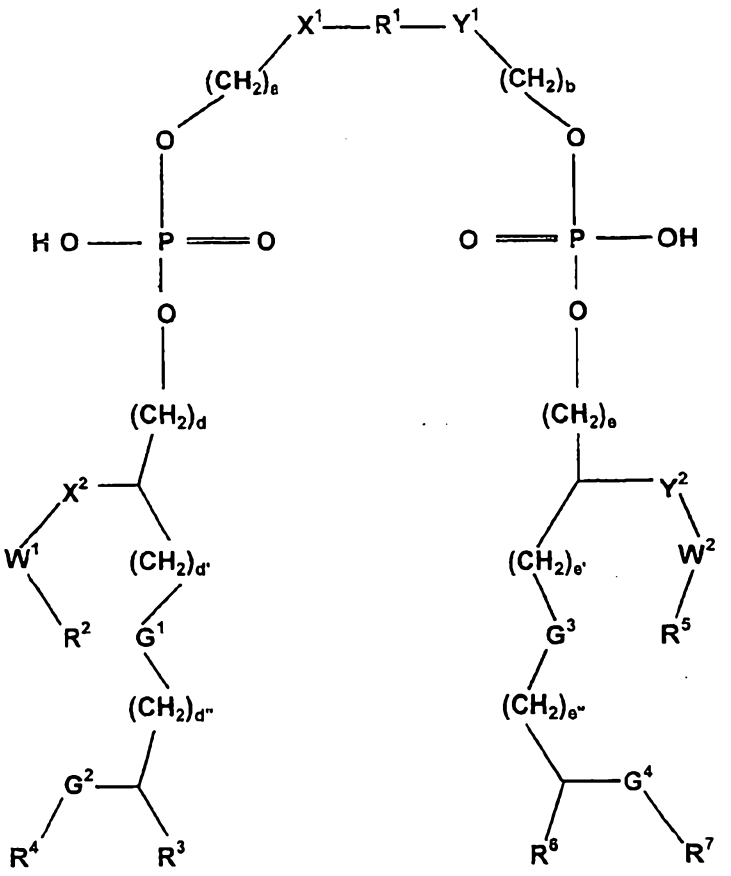
Depending on the devices used, the measurements may vary slightly according to the components of the device and the data processing software used.

The phase inversion temperature of a thermoreversible emulsion according to the invention is a characteristic specific to each emulsion and varies according to the nature of its components and to their relative concentrations. Advantageously, the composition of the emulsion according to the invention is chosen such that the phase inversion occurs at a temperature of between 45°C and 80°C preferably of between 50 and 65°C. This temperature range is advantageous because there is no risk of the emulsion changing state if it is stored at a relatively high temperature (≈37°C). Furthermore, as in the method for preparing the thermoreversible emulsion, the heating of the components does not exceed 80°C, which contributes to maintaining the structure and integrity of the components and in particular of the TLA4. When the phase inversion temperature of the emulsion is high, in particular when it is greater than or close to 80°C, it may be useful to lower it by adding to the composition of the emulsion an alditol, which is usually chosen from sorbitol, mannitol, glycerol, xylitol or erythritol. When the alditol is used in a concentration range of from 0.1 to 10% (w/w), preferably in a concentration range of from 1 to 10% (w/w) and in particular in a concentration range of from 2 to 7% (w/w), the phase inversion temperature of the emulsion can be decreased by approximately 10°C. The phase inversion temperature of the emulsion can also be decreased by

replacing the aqueous phase consisting only of water with a buffered saline aqueous phase. A TRIS buffer, a phosphate buffer such as PBS, the Dulbecco PBS buffer without Ca^{2+} or Mg^{2+} or a citrate buffer is normally used.

The chemical compounds of formula I, II, III or IV are obtained by synthesis according to the processes described in particular in US 2003/0153532 or in US 2005/0164988.

In particular, the TLA4 according to the invention is a chemical compound of formula I,



or a pharmaceutically acceptable salt of this compound.

Preferably,

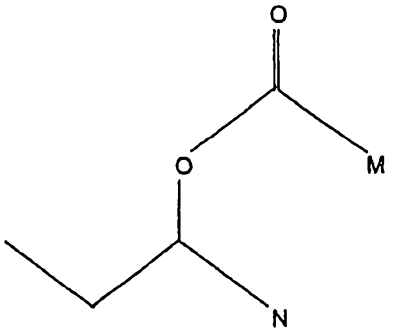
R1 is C(O) or C(O)-$(CH2)_n$-C(O), n being 1, 2, 3 or 4,

a, b, d, d', d", e, e' and e" are independently 1 or 2,

X1, X2, Y1 and Y2 are NH,

W1 and W2 are C(O),

R2 and R5 are independently selected from the group consisting of a C_{10}-C_{15} straight chain alkyl optionally substituted with an oxo, an NH-(C_{10}-C_{15} straight chain alkyl), and



in which M and N are independently a C_2 to C_{20} straight chain alkyl or alkenyl,

R3 and R6 are C_5-C_{10} straight chain alkyls,

R4 and R7 are selected from the group consisting of a hydrogen, C(O)-(C_8-C_{12} straight chain alkyl) or C(O)-(C_8-C_{12} straight chain alkenyl),

G1 and G3 are an oxygen or –NH(CO)-,

G2 and G4 are an oxygen.

TLA4 exerts an immunostimulting activity *in vitro* and/or *in vivo*. The *in vitro* immunostimulating activity is evaluated in particular:

1) by measuring the increase in the production of TNFα by the cells of whole human blood, or
2) by measuring the increase in the production of alkaline phosphatase by a THP-1 line transfected with the alkaline phosphatase gene under the control of the TNFα promoter, or
3) by measuring the increase in the production of cytokines such as IL-10 and interferon γ by murine splenocytes, or
4) by measuring the increase in the production of TNFα by the murine macrophage line RAW264, or
5) by measuring the increase in the production of IL-6 by the U373 human astrocytoma, or

6) by measuring the increase in the activation/maturation of dendritic cells derived from human monocytes, on the basis of the expression of activation markers such as CD25, CD80/CD83, by flow cytometry.

All these measurement assays are well known to those skilled in the art and are in particular described in example 7 of US 2003/0153532 or in the Journal of Biological Chemistry, (2001), vol 276/3, page 1873-1880.

The *in vivo* immunostimulating activity is reflected by an increase in the humoral response and/or in the specific cell response. To evaluate the humoral response, the production of specific antibodies directed against an antigen is measured. By way of example, reference may be made to the assays which are described in example 8 of US 2003/0153532 for evaluating this response. When the production of specific antibodies (within the form of total immunoglobulins or of a specific isotype) observed following the injection of a TLA4-related antigen is greater than that which is observed subsequent to the administration of the same amount of antigen alone, TLA4 is considered to exert an *in vivo* immunostimulating activity. The immunostimulating activity of TLA4 can also be evaluated using assays for measuring the specific cellular response, which are well known to those skilled in the art, for instance by measuring the activity of cytotoxic T lymphocytes (CTLs) or lymphoproliferation.

Preferably, TLA4 is chosen from the group consisting of the chemical compounds identified and described in US 2003/0153532 under the names ER803022, ER803058, ER803732, ER803789, ER804053, ER804057, ER804058, ER804059, ER804442, ER804764, ER111232, ER112022, ER112048, ER 112065, ER112066, ER113651, ER118989, ER119327 and ER119328.

The compounds can be in the form of diastereoisomers or in a racemic form (mixture of diastereoisomers) when the chemical structure comprises several asymmetrical carbons. For example, ER804057 and ER804053, which have 4 asymmetrical carbons, are diastereoisomers of ER112066, which is the racemic form. ER804057 is in an (R,R,R,R)-type isomeric configuration, whereas ER804053 is in an (R,S,S,R)-type configuration. Similarly, ER804058, which is in an (R,R,R,R)-type isomeric configuration, and ER804059, which is in an (R,S,S,R)-type isomeric

configuration, are diastereoisomers of ER113651, which is the racemic form. ER803022, which is in an (R,R,R,R)-type configuration, ER803732, which is in an (R,S,S,R) configuration, and ER803789, which is in an (R,R,S,R) configuration, are also diastereoisomers of one and the same chemical molecule. The diastereoisomers which have an R,R,R,R-type configuration, which are generally more active than the other forms, are preferably used. Among these, ER804057 is particularly preferred. It is dodecanoic acid *(1R,6R,22R,27R)*-1,27-diheptyl-9,19-dihydroxy-9,19-dioxido-14-oxo-6,22-bis[(1,3-dioxotetradecyl)amino]-4,8,10,18,20,24-hexaoxa-13,15-diaza-9,19-di-phosphaheptacosane-1,27-diyl ester; it is in the form of a free acid or in the form of a salt. The molecular weight of the free acid form is 1579, that of the disodium salt is 1624. The empirical formula of the disodium salt is $C_{83}H_{158}N_4Na_2O_{19}P_2$.

From a structural point of view, the TLR4 agonist according to the subject of the invention is an amphiphilic molecule. Amphiphilic molecules have a behavior that is both hydrophilic and hydrophobic and have a tendency to precipitate over time. They often dissolve incompletely in organic or aqueous solvents and are often the cause of unstable solutions or solutions that are difficult to reproduce. There exists a need to improve the formulation of these molecules. The emulsion as described in the invention satisfies this need by providing emulsions which are stable over time. An emulsion according to the invention which is stored for 6 months at +4°C conserves the characteristics that it initially had: the size distribution of the oil droplets does not substantially vary; the milky, fluid and homogeneous aspect of the invention is conserved; and, manifestly, the structural integrity of the TLA4 is not impaired, as shown in example II. It has even been noted that an emulsion according to the invention can be stored at a temperature of -2°C for at least 48 hours without any notable variation being observed in terms of the oil droplet size distribution. Moreover, the emulsion according to the invention decreases the pyrogenic capacity of certain TLR4 agonists.

The ratio of the amount of TLA4 to the total amount of hydrophilic and hydrophobic surfactants of the emulsion is usually between 0.01×10^{-2} and 5×10^{-2}, preferably between 0.1×10^{-2} and 2×10^{-2}. In this ratio range, the amount of TLA4 is sufficiently small so as not to exert any influence over the emulsifying capacity of the

surfactants, but it is present in sufficient amount to exert an immunostimulating activity *in vitro* and/or *in vivo*.

The hydrophilic surfactant according to the invention has an HLB (hydrophilic/lipophilic balance) ≥ 10 and belongs to the polyoxyethylene alkyl ether (PAE) chemical group, also called polyoxyethylenated fatty alcohol ethers. These nonionic surfactants are obtained by chemical condensation between a fatty alcohol and ethylene oxide. They have a general chemical formula of the type $R-(O-CH_2-CH_2)_n-OH$ in which the radical R usually denotes a saturated or unsaturated alkyl residue and n denotes the number of ethylene oxide units. According to the subject of the invention, R contains between 1 and 50 carbon atoms, preferably between 4 and 20 carbon atoms, and particularly preferably between 10 and 20 carbon atoms. n is ≥ 2, generally between 4 and 50. The emulsion according to the invention usually comprises a single hydrophilic PAE. A mixture of several PAEs is also suitable provided that the overall HLB is ≥ 10.

The polyoxyethylenated fatty alcohol ethers that are suitable for the subject of the invention can be in a form that is liquid or solid at ambient temperature. Among the solid compounds, preference is given to those which dissolve directly in the aqueous phase or which do not require substantial heating.

Insofar as the number of ethylene oxide units is sufficient, the polyoxyethylenated ethers of lauryl alcohol, myristyl alcohol, cetyl alcohol, oleyl alcohol and/or stearyl alcohol are particularly suitable for the subject of the invention. They can in particular be found in the range of products known under the trade names Brij®, Eumulgin® or Simulsol®.

A particularly preferred emulsion according to the invention contains, as nonionic hydrophilic surfactant, a polyoxyethylene alkyl ether chosen from the group consisting of polyoxyethylene (12) cetostearyl ether (ceteareth-12) (sold under the name Eumulgin® B1), polyoxyethylene (20) cetostearyl ether (ceteareth-20) (Eumulgin® B2), polyoxyethylene (21) stearyl ether (steareth-21) (Eumulgin® S21), polyoxyethylene (20) cetyl ether (ceteth-20) (Simulsol® 58 or Brij® 58), polyoxyethylene (10) cetyl ether

(ceteth-10) (Brij® 56), polyoxyethylene (10) stearyl ether (steareth-10) (Brij® 76), polyoxyethylene (20) stearyl ether (steareth-20) (Brij® 78), polyoxyethylene (10) oleyl ether (oleth-10) (Brij® 96 or Brij® 97) and polyoxyethylene (20) oleyl ether (oleth-20) (Brij® 98 or Brij® 99). The number displayed next to each chemical name corresponds to the number of ethylene oxide units in the chemical formula.

A compound that is particularly suitable and preferred owing to its semisynthetic origin is Eumulgin™ B1 (ceteareth-12) provided by the company Cognis.

The emulsion according to the invention also contains a nonionic hydrophobic surfactant, the HLB of which is ≤ 6. The emulsion usually comprises a single nonionic hydrophobic surfactant. A mixture of several nonionic hydrophobic surfactants is also suitable provided the overall HLB is ≤ 6. Typically, it involves a hydrophobic sorbitan ester or a hydrophobic mannide ester. Sorbitan esters are usually obtained by an esterification reaction between a fatty acid and sorbitol, sorbitol monoanhydride or sorbitol dianhydride. Mannide esters are normally obtained by an esterification reaction between a fatty acid and mannitol monoanhydride or dianhydride. Preferably, it involves mannide monooleate (sold by the company Sigma or supplied by the company Seppic under the name Montanide 80™) or sorbitan monooleate (sold under the name Span® 80, Dehymuls SMO™ (Cognis) or Montane 80™ (Seppic)).

By virtue of the selection of these particular surfactants among all the surfactants proposed in the prior art for preparing emulsions, it has now been found that an adjuvant O/W emulsion can very advantageously be produced using a phase inversion method that is easy to implement.

When the respective concentrations of hydrophilic and hydrophobic surfactants are such that the HLB of the mixture (HLB_m is between 8.5 and 10, and more particularly between 8.6 and 9.6, the emulsions are generally homogeneous and often at least 90% of the population by volume of the oil droplets has a size ≤ 0.2 μm . These emulsions are, moreover, particularly stable. The amounts of hydrophilic and hydrophobic surfactants in the squalene emulsion are preferably adjusted such that the HLB_m is between 8.5 and 10, and more particularly such that the HLB_m is between 8.6

and 9.6. To determine the respective concentrations of hydrophilic and hydrophobic surfactants in the composition of the emulsion, the following formula is used:

$HLB_m = HLB_e \times M + HLB_{pae} \times (1-M)$ in which,

HLB_m corresponds to the HLB of the mixture, which is preferably between 8.5 and 10, and more particularly between 8.6 and 9.6.
HLB_e corresponds to the HLB of the hydrophobic surfactant.
M corresponds to the percentage by weight of the hydrophobic surfactant in the mixture made up of the hydrophobic surfactant and PAE.
HLB_{pae} corresponds to the HLB of the PAE.

Squalene, which represents the oily phase of the emulsion, has the empirical chemical formula $C_{30}H_{50}$ and comprises 6 double bonds. This oil is metabolizable and has the required qualities to be used in an injectable pharmaceutical product. It comes from shark liver (animal origin) but can also be extracted from olive oil (plant origin). Good results have in particular been obtained using the squalene provided by the company Fluka, which is of animal origin. Generally, the amount of squalene represents between 5 and 45% of the total weight of the emulsion.

The ratio by mass of the amount of squalene to the total amount of surfactants in the emulsion according to the invention is usually between 2.0 and 4.0, preferably between 2.5 and 3.5.

A composition of the emulsion according to the invention that is particularly preferred comprises:

- squalene,
- a phosphate buffer or a citrate buffer as aqueous solvent,
- the compound ER 804057 as TLR4 agonist,
- ceteareth-12 (Eumulgin®B1) as hydrophilic surfactant,
- sorbitan monooleate as hydrophobic surfactant.

The amount of squalene represents between 5 and 45% of the total weight of the emulsion. The amount of the compound ER804057 usually represents between 0.05% and 2% of the weight of the two surfactants. Preferably, the amounts of ceteareth-12 and of sorbitan monooleate are such that the HLB of the mixture of the two surfactants is between 8.5 and 10, and more particularly between 8.6 and 9.6. The ratio of the amount of squalene to the total amount of ceteareth-12 and of sorbitan monooleate is between 2.0 and 4.0, preferably between 2.5 and 3.5. Furthermore, this composition can contain mannitol, the amount of which usually represents between 0.1% and 10% of the total weight of the emulsion.

The aqueous phase of the emulsion according to the invention can also contain a lyophilization substrate containing one or more cryoprotective agents. The cryoprotective agents are usually chosen from sugars such as sucrose, polyalcohols such as mannitol or sorbitol, or sugar derivatives such as alkylpolyglycosides, for instance sodium decyl-D-galactoside uronate or dodecyl β-maltoside. A lyophilization substrate which contains sucrose, mannitol and dodecyl β-maltoside as a mixture is normally used. The emulsion according to the invention can then be lyophilized and conserved in the form of a lyophilizate. It conserves, however, all its characteristics since, once it is taken up in an aqueous phase, it again becomes a milky, stable and fluid thermoreversible O/W emulsion with an oil droplet size distribution similar to that which preexisted before lyophilization.

The emulsion according to the invention also plays the role of adjuvant of the immune response to an antigen. For the purpose of the present invention, the term "antigen" is intended to mean any antigen which can be used in a vaccine, whether it is a living, attenuated or killed whole microorganism, an extract of a microorganism or a subunit form. When it is in a subunit form, the nature of the antigen is of little importance: it may be a peptide, a protein, a glycoprotein, a polysaccharide, a glycolipid, a lipopeptide or a nucleic acid. Among the antigens that are suitable for the subject of the invention, mention is made of the bacterial antigens originating from *Clostridium tetani*, from *Clostridium diphtheriae*, from *Bordetella pertussis*, from *Haemophilus influenzae type b*, from *Streptococcus pneumoniae*, from *Neisseria meningitidis*, from

Shigella sp., from *Salmonella typhi*, from *Staphylococcus aureus* or *Staphylococcus epidermidis*, from *Mycobacterium tuberculosis*, from *Chlamydia trachomatis* or from *Streptococcus sp*, viral antigens originating from the hepatitis A, B or C virus, from the flu virus, from the respiratory syncytial virus, from the West Nile virus, from the rabies virus, from the poliovirus, from the HIV virus, from the dengue virus, from the Japanese encephalitis virus, from the yellow fever virus, from the cytomegalovirus or from the herpes virus, from parasitic antigens originating in particular from *Plasmodium sp.* or from tumor antigens. These antigens can be obtained using genetic recombination methods or using extraction methods well known to those skilled in the art. The emulsion according to the invention acts on humoral immunity by increasing the production of specific antibodies and/or on specific cellular immunity by promoting in particular T lymphocyte proliferation, the development of a specific cytolytic response (CTL response) and/or the production of cytokines, chemokines and growth factors, produced by the activated lymphocytes.

For this reason, a subject of the invention is also the use of an emulsion according to the invention, for preparing a vaccine composition. The vaccine composition obtained proves to be more immunogenic, for example because the composition induces a greater specific immune response, whether it is of humoral type and/or of cellular type, or because a smaller amount of antigen is necessary in order to obtain an immune response of the same intensity and of comparable duration. The vaccine composition obtained from an emulsion according to the invention can be administered by any of the routes normally used or recommended for vaccines: parenterally, intradermally, subcutaneously, intramuscularly or mucosally, and can be in various forms, in particular liquid or lyophilized. It can be administered by means of a syringe or by means of a needle-free injector for intramuscular, subcutaneous or intradermal injection, or by means of a nasal spray.

The vaccine composition is generally in the form of a mixture of the antigen with an emulsion according to the invention. It may also be in the form of an extemporaneous formulation. In this case, the antigen and the emulsion are brought into contact just before or at the time of the administration of the vaccine composition. For example, the antigen can be lyophilized and taken up with the emulsion just before administration or,

conversely, the emulsion can be in a lyophilized form and taken up with a solution of the antigen. The vaccine composition can also be in a specific injection device such as the "bypass" syringe when it is desired not to mix the antigen with the emulsion.

When the vaccine composition is in the form of a mixture obtained by dilution of an emulsion according to the invention with an antigen solution, it is usually in the form of an O/W emulsion in which the amount of squalene in general represents by weight between 0.5 and 5% of the total weight of the composition. It can also be in the form of a thermoreversible O/W emulsion when the amount of squalene in the vaccine composition reaches or exceeds 5% (w/w). When the vaccine composition is a thermoreversible O/W emulsion, it can in particular be in a form in which at least 90% of the population by volume of the oil droplets has a size ≤ 0.2 μm.

Surprisingly, the emulsion according to the invention has a greater ability to induce neutralizing antibodies than an O/W emulsion of the prior art obtained by microfluidization, the composition of which contains squalene, polyoxyethylene sorbitan monooleate (Tween®80) and sorbitan trioleate (Span® 85) (O/W emulsion of the prior art).

Neutralizing antibodies are functional antibodies directed against an infectious microorganism, produced by an individual who has been immunized with an antigen related to or derived from this microorganism or who has been in contact with this microorganism, and which prevents infections of the cells by this microorganism. They play a very important role in the prevention or treatment of infections caused by intracellular microorganisms, in particular viruses and single-cell parasites, in particular plasmodium sp.. Antigens originating from the "sporozoite" form of *Plasmodium falciparum*, (such as the major surface protein of the sporozoite (circumsporozoite protein), LSA3, or the Pfs 16 antigen), and antigens originating from the "merozoite" form of *Plasmodium falciparum* (such as the MSP1, MSP2, MSP3, EBA-175, Rhop-1, Rhop-2, Rhop-3, RAP-1, RAP-2, RAP-3, Pf155/RESA or AMA-1 antigen) induce neutralizing antibodies. The use of an emulsion according to the invention for preparing a vaccine composition containing one or more antigens derived from *Plasmodium falciparum* sporozoites or merozoites is indicated for amplifying the neutralizing

immune response. An emulsion according to the invention can in particular be used to prepare a vaccine composition comprising, as vaccine antigen, the *Plasmodium falciparum* LSA3 protein. The gene encoding this protein was identified by Gardner et al., (Science (1998), 282, 1126-1132) and is on chromosome 2 of *Plasmodium falciparum* (strain 3D7). The complete sequence of the gene is 12240 base pairs long and encodes a 1558 amino acid protein. The nucleotide and protein sequences are described in the EMBL databank under accession number AE001424 and uniprot number 096275-PLAF7. The whole protein as described, peptides, or fragments of this protein, such as those described in WO 02/38176, can be used as vaccine antigen. The whole protein (which may contain one or more point mutations so as to take into account the variations that exist between strains of *Plasmodium falciparum*) or a fragment of this protein, the amino acid sequence of which has at least 80% identity with respect to the whole sequence described in uniprot 096275-PLAF7, is normally used. The effectiveness of certain antiviral vaccines is, in certain cases, evaluated on the basis of the titer of neutralizing antibodies that they induce. This is the case of the flu vaccine, the effectiveness of which is related to the titer of hemagglutination-inhibiting (HAI) antibodies.

The emulsion according to the invention is used to prepare a vaccine composition for the treatment or prevention of infectious diseases in humans or animals (birds, horses) linked to the flu virus. Depending on the nature of the flu vaccine, the vaccine composition can be in various forms:

- When the flu vaccine contains one or more inactivated whole or "split" virus strains, or is in the form of a subunit vaccine containing purified hemagglutinin from one or more viral strains, or in the form of virosomes (Berna vaccine), the vaccine composition is usually in the form of a mixture, of an O/W emulsion or of a thermoreversible O/W emulsion.

- When the flu vaccine contains one or more live attenuated virus strains, the vaccine composition is preferably in a device, of the bypass syringe type, such that the live virus is not in direct contact with the emulsion. The viral suspension and emulsion according to the invention are in two different compartments of the syringe.

The flu vaccines are manufactured from flu viruses cultivated in eggs or in cells according to methods well known to those skilled in the art, and all comprise, as an essential component, the hemagglutinin of one or more virus strains.

A subject of the invention is therefore also the use of an emulsion according to the invention, for preparing a vaccine composition comprising, as vaccine antigen, one or more flu virus hemagglutinins. This vaccine composition can be used to immunize:

1) populations of individuals who are seronegative with respect to the flu virus: these are individuals who have never been in contact or sensitized with the flu virus or its immunogenic components, or individuals who have never been in contact with a new strain of flu virus responsible for a pandemic;
2) populations of individuals who are seropositive with respect to the flu virus: these are individuals who have already been in contact or sensitized with the flu virus or its immunogenic components;
3) populations of elderly individuals who commonly exhibit an impairment of cellular and/or humoral immunity, which is observed in particular with respect to the flu virus.

The emulsion according to the invention is also used to prepare a vaccine composition for the treatment or prevention of infectious diseases caused by herpes virus (HSV1, HSV2, cytomegalovirus (CMV)). The viral envelope antigens are generally used in the vaccine composition. In CMV infections, the antibodies which are directed against the viral envelope proteins, mainly glycoprotein B (gB) and glycoprotein H (gH), and which neutralize the viral infection, play a very important role in the development of a protective immunity. The use of an emulsion according to the invention in the preparation of a vaccine composition containing a CMV envelope protein has the effect of increasing the production of neutralizing antibodies.

A subject of the invention is therefore the use of an emulsion according to the invention, for preparing a vaccine composition comprising, as vaccine antigen, a CMV envelope antigen. Typically, the antigen is the gB glycoprotein and/or the gH

glycoprotein. It may also be a peptide or a polypeptide derived from gB and/or from gH, comprising one or more neutralizing epitopes.

gB in its native form (gp130), encoded by the UL 55 gene of CMV, is a glycoprotein of 906 or 907 amino acids, depending on whether the AD169 strain or the Towne strain is involved. The protein sequences of these two strains are described in US 2002/0102562 (figure 2). The native form of gB contains a signal sequence followed by an extracellular domain containing an endoproteolytic cleavage site between residues arginine 460 and serine 461, by a transmembrane domain and by an intracellular domain. Several antigenic domains inducing neutralizing antibodies have been described. This involves in particular the domain located between amino acid residues 461 and 680 of gp 130, this domain being subdivided into two discontinuous domains, the domain between residues 461 and 619 and the domain between residues 620 and 680 (US 5,547,834). It also involves the AD-1 domain located between amino acid residues 552 and 635 or the AD-2 domain located between amino acid residues 50 and 77 (Journal of General Virology (1999), 80, 2183-2191; Journal of Virology (2005), 79, 4066-4079). Consequently, a polypeptide which comprises, in its amino acid sequence, a sequence homologous to one of the domains mentioned is suitable for the subject of the invention. Typically, the polypeptide comprises, in its amino acid sequence, a sequence homologous to that which is located between residues 461 and 680 of gp 130, or more specifically to that which is located between residues 552 and 635. The term "homologous sequence" is intended to mean any amino acid sequence which has at least 80% identity with the amino acid sequence of the antigenic domain under consideration located on gp 130 of the Towne or AD 169 strain (described in US 2002/0102562). Typically, the sequence homology is based on an identity of at least 90%, and even more particularly, the sequence homology is based on a sequence identity of 100%.

Among the gB-derived peptides or polypeptides that are suitable for the subject of the invention, mention is in particular made of gp 55 as described in US 5,547,834. It is derived from the cleavage of gB at the endoproteolytic cleavage site; its amino acid sequence corresponds to that which is between serine residue 461 and the C-terminal end. Truncated forms of gp 55 can also be used, such as a gp 55 depleted of all or part of the transmembrane sequence and of all or part of the intracellular C-terminal domain

(for example, a peptide having a sequence homologous to the amino acid sequence of gp130 between residues 461 and 646) or a gp 55 depleted of all or part of the intracellular C-terminal domain (for example, a peptide having a sequence homologous to the amino acid sequence of gp130 between residues 461 and 680) which are described in US 5,547,834. It is also possible to use a mutated form of gB which carries one or more mutations at the endoproteolytic cleavage site such that the latter is made ineffectual. The mutation(s) is (are) located between residues 457 and 460 of the sequence of gp130, and more particularly is (are) located at arginine 460 and/or lysine 459 and/or arginine 457. A CMV envelope antigen that is particularly suitable for the subject of the invention is a truncated form of gB depleted of all or part of the C-terminal domain and/or depleted or all or part of the transmembrane sequence and in which the cleavage site is ineffectual. A truncated form of gB which is particularly preferred corresponds to that which is described in US 6,100,064, called gBdTM; it carries three mutations at the cleavage site and a deletion in the transmembrane region between amino acid residues valine 677 and arginine 752, such that the extracellular domain is directly connected to the cytoplasmic domain.

The gB protein or the peptides or polypeptides derived therefrom are obtained by means of genetic recombination methods and purified according to methods well known to those skilled in the art. The methods described in US 6,100,064 and in US 2002/0102562, incorporated by way of reference, can in particular be used. To increase their immunogenicity, they can secondarily be conjugated to a carrier protein or fused to other proteins, in particular to particle-forming proteins such as the hepatitis B surface antigen (HbS). The gB protein or the peptides derived therefrom can also be expressed by recombinant viruses, in particular by recombinant adenoviruses or recombinant poxviruses. To prepare these recombinant vectors expressing gB or derived peptides, the methods which are described in particular in US 6,162,620, US 5,866,383, US 5,552,143, US 6,183,750, US 5,338,683 or WO 9215672 or in WO 9639491 are used. gB can also be provided by a strain of CMV which has been attenuated by successive passages on cell cultures, in particular the Towne strain which has already been tested for vaccine purposes.

The gH protein is encoded by the *UL 75* gene of CMV. It is a glycoprotein of 742 or 743 amino acids depending on whether the strain is the Towne strain or the AD169 strain. The sequences are described in US 5,474,914 (figure 1) and in US 6,610,295 (figure 5(a)). The protein sequence of gH deduced from its nucleotide sequence contains a signal peptide followed by an extracellular domain that does not have an endoproteolytic cleavage site, by a transmembrane domain and by a C-terminal cytoplasmic domain. The neutralizing epitopes are in the extracellular domain, mainly in the N-terminal portion of this domain, more specifically between amino acid residues 15 and 142 of the protein sequence of native gH, and even more specifically between amino acid residues 33 and 142. A major neutralizing epitope of the AD 169 strain has been identified and is between residues 33 and 43 of the sequence of gH, and has the sequence LDPHAFHLLL (Urban M et al.: J. Virol (1992, vol 66/3, p1303-1311)). Consequently, a polypeptide which comprises, in its amino acid sequence, a sequence homologous to the sequence LDPHAFHLLL or a sequence homologous to that which is between residues 15 and 142 or between residues 33 and 142 of the protein sequence of gH is suitable for the subject of the invention. The term "homologous sequence" is intended to mean an amino acid sequence which has at least 80% identity with the amino acid sequence which is either between residues 15 and 142 or between residues 33 and 142 of the protein sequence of gH of the AD 169 strain, or with the sequence LDPHAFHLLL. More particularly, the sequence homology is based on an identity of at least 90%, and even more particularly the sequence homology is based on a sequence identity of 100%.

As gH-derived peptides or polypeptides that are suitable for the subject of the invention, mention is made of gH depleted of all or part of its transmembrane region and/or depleted of all or part of its cytoplasmic region. Typically, this corresponds to a gH protein from which at least 5 C-terminal residues, preferably at least 10 C-terminal residues, and even more preferably between 20 and 34 residues of the C-terminal end of the amino acid sequence have been deleted.

The gH protein and the polypeptides or the peptides derived therefrom are obtained by means of genetic recombination methods and purified according to methods well known to those skilled in the art, in particular those described in US 5,474,914 or in US 5,314,800, incorporated by way of reference. To increase their immunogenicity, they

can be secondarily conjugated to a carrier protein. They can also be produced in the form of fusion proteins, as described in J. Virol (1992, vol 66/3, p1303-1311). The gH protein and the polypeptides and the peptides derived therefrom can also be expressed by recombinant viruses, in particular by recombinant adenoviruses or recombinant poxviruses. To prepare these recombinant vectors expressing gH or derived forms, the methods which are described in particular in US 6,162,620, US 5,866,383 or US 5,552,143, or in WO 9639491 are used. The gH protein can also be provided by a strain of CMV which has been attenuated by successive passages on cell cultures, in particular the Towne strain which has already been tested for vaccine purposes.

It is also possible to use, as vaccine antigen, a protein resulting from fusion between the gB glycoprotein or the gH glycoprotein (or a truncated form of said glycoproteins) and a membrane protein of HSV1 or of HSV2 (or a truncated form thereof). By way of example, mention is made of the fusion proteins described in EP 0759995, in particular gB 685* and gB 685** which result from fusion between a portion of the CMV gB glycoprotein and a portion of the HSV gD glycoprotein.

Depending on the nature of the CMV antigen, the vaccine composition can be in various forms:

- When the antigen is a protein or a peptide, the vaccine composition can be in the form of a mixture, of an O/W emulsion or of a thermoreversible O/W emulsion. It can also be in the form of an extemporaneous preparation which is prepared just before administration. The vaccine composition can also be inside a device, such as a "bypass" syringe which physically separates the antigen from the emulsion.
- When the CMV antigen is in the form of a recombinant virus expressing gB, gH or a peptide derived from gB or gH, or when it is in the form of an attenuated strain of a CMV, the antigen and the emulsion according to the invention are not usually directly in contact in the vaccine composition. The antigen and the emulsion can be inside a device which physically separates them, such as a "bypass" syringe, but they are administered at the same time at the same site of administration.

The emulsion according to the invention also orients the specific CD4+ T cell response toward a Th1 profile by promoting the production of Th1 cytokines (IL2,

IFN-γ, etc.) and/or by decreasing the production of Th2 cytokines (IL4, IL5, IL10, etc.) in response to an antigen presented in an MHC class II context. This effect is evaluated by measuring the amount of IFN-γ and IL5 produced after restimulation *in vitro* with an antigen related to that which was used for the *in vivo* immunization and by determining the IFN-γ/IL5 ratio. The higher the ratio, the more the CD4+ response tends toward a Th1 type. The CD4+ T cell response profile can also be evaluated indirectly by measuring the ratio between the titer of specific Ig2as/specific IgG1s obtained after immunization of mice with a vaccine composition according to the invention.

The emulsion according to the invention can therefore be used to correct an imbalance in the CD4+ T cell response which is observed in certain populations of individuals who show an immune deficiency or an impairment of the immune system. These are in particular elderly individuals who show a deficiency in production of IFN-γ and/or IL2 subsequent to an *in vitro* stimulation with antigens originating from intracellular microorganisms, in particular with a flu antigen (Ouyang et al. (Mechanisms of ageing and development), 2000, vol. 121, 131-137).

The subject of the invention is therefore the use of an emulsion, according to the invention, for preparing a vaccine composition intended for a population of individuals who show an imbalance in the CD4+ T cell response.

A subject of the invention is also a method for preparing an O/W emulsion according to the invention, comprising a step in which a W/O inverse emulsion is obtained by raising the temperature and a step in which the W/O inverse emulsion is converted to an O/W emulsion by lowering the temperature. This conversion takes place when the W/O emulsion obtained is lowered to a temperature below the phase inversion temperature of this emulsion.

According to one embodiment of the method, the W/O emulsion is obtained by carrying out a first step in which an aqueous phase comprising an aqueous solvent, a polyoxyethylene alkyl ether and a TLR4 agonist is mixed with an oily phase comprising squalene and a nonionic hydrophobic surfactant so as to obtain an O/W emulsion, and a

second step in which the O/W emulsion is heated to a temperature which is at least the phase inversion temperature of the emulsion.

The aqueous phase comprising the aqueous solution (usually a buffered solution), the TLR4 agonist (if it is not in the oily phase) and the nonionic hydrophilic surfactant is incorporated into the oily phase comprising the squalene, and the nonionic hydrophobic surfactant, or vice versa: the oily phase is incorporated into the aqueous phase. This incorporation is carried out with mechanical stirring. A noncalibrated, unstable coarse O/W emulsion is obtained (preemulsion). This preemulsion is heated with mechanical stirring until phase inversion is obtained, i.e. a W/O emulsion is obtained. The phase inversion or transition can be followed by conductimetry. The temperature at which the curvature change reflecting the passage from one type of emulsion to another occurs is the phase inversion temperature. In reality, this temperature is a temperature range rather than a very specific point value; in fact, it may be considered that this temperature is capable of varying by one or two degrees, so that the entire emulsion undergoes the phase inversion phenomenon. When the emulsion is in the form of a W/O emulsion, an abrupt drop in the conductivity is observed. The heating is stopped and the mixture is cooled. The cooling can be carried out passively, by simply allowing the temperature to return spontaneously to ambient temperature, or more actively, by, for example, immersing the emulsion in an ice bath. During the decrease in temperature, the W/O emulsion will again inverse at the phase inversion temperature so as to again give an O/W emulsion. The emulsion can be stored as it is while awaiting dilution with a solution comprising the vaccine antigen. It is thermoreversible, which means that, if it is again brought to a temperature at least equal to the phase inversion temperature, it will again become a W/O emulsion. The phase inversion temperature is usually between 45 and 80°C, and typically between 50 and 65°C. The components of the emulsion, in particular the TLR4 agonist, are thus subjected to moderate heating which prevents evaporation of the aqueous phase or chemical degradation of the components.

According to another embodiment, the W/O emulsion is obtained by separately heating an aqueous phase comprising an aqueous solvent, a polyoxyethylene alkyl ether and a TLR4 agonist and an oily phase comprising squalene and a nonionic hydrophobic surfactant, at a temperature which is at least equal to the phase inversion temperature of

the emulsion, and then by mixing the aqueous phase with the oily phase while at the same time keeping the temperature of the mixture at a temperature which is at least equal to the phase inversion temperature.

In this case, the aqueous and oily phases are heated separately to a temperature slightly above the phase inversion temperature, before mixing them to give a W/O inverse emulsion, which will subsequently be cooled until the submicronic O/W emulsion is obtained. These operations can be carried out in separate containers for a batch preparation.

It is also possible to use an on-line manufacturing method. The method consists in mixing, under hot conditions, the two aqueous and oily phases prepared separately, through a thermostatted static mixer, followed by on-line cooling through a refrigerated heat exchanger connected at the outlet of the static mixer, and then by the final recovery of the emulsion according to the invention in an appropriate container (flask or reactor). A static mixer consisting of a succession of mixing elements composed of crossed blades that are sloping with respect to the axis of the tube into which they are introduced has been successfully used. The energy required for the mixing is provided by the pumps which convey the fluids and the mixing is carried out without moving parts, through the mixing elements by successive separation, displacement and combination of the constituents of the mixture.

The on-line manufacturing method is carried out in the following way: the aqueous phase and the oily phase are prepared separately, as above, in two flasks or reactors. The two phases are heated with stirring to a temperature slightly above the phase inversion temperature. The two phases are then introduced into a thermostatted static mixer by means of 2 pumps, the flow rates of which are regulated so as to obtain the composition of the emulsion according to the invention. The W/O inverse emulsion is obtained during the passage of the two phases through the static mixer. The inverse emulsion is subsequently cooled by on-line passage through a refrigerated heat exchanger connected at the outlet of the static mixer. The W/O emulsion will then inverse through the refrigerated heat exchanger to give rise to an O/W emulsion, which

will be collected in a flask or reactor and the characteristics of which are identical to those of the emulsion obtained by means of a batch method.

Alternatives to the embodiments of the method which have just been described exist; when the behavior or the TLR4 agonist is more hydrophobic than hydrophilic, it is introduced into the oily phase rather than into the aqueous phase. The TLR4 agonist can also be introduced after the mixing of the oily phase and the aqueous phase has been carried out, or when the emulsion has already been heated and it is in a W/O emulsion form. The aqueous phase may also contain an alditol. Finally, the method for preparing the emulsion according to the invention may comprise several successive thermoinversion cycles.

A subject of the invention is also a method for preparing a vaccine composition, in which at least one vaccine antigen is mixed with an O/W emulsion containing a TLR4 agonist, the chemical structure of which does not comprise a sugar ring, characterized in that the O/W emulsion containing the TLR4 agonist has been prepared according to a phase inversion method comprising a step in which an emulsion is obtained in the form of a W/O inverse emulsion by increasing the temperature and a step in which the W/O emulsion is converted to an O/W emulsion by lowering the temperature.

A simple embodiment consists in mixing an aqueous solution of a vaccine antigen into a thermoreversible O/W emulsion obtained according to one of the embodiments which have just been described. The vaccine composition obtained is in the form of an O/W emulsion or in the form of a thermoreversible O/W emulsion when the amount of squalene represents by weight at least 5% of the total weight of the vaccine composition. Alternatively, the antigen may be mixed with the aqueous phase or with the oily phase before preparing the emulsion. Carrying out the procedure in this way of course implies that these are antigens which are compatible with the thermoinversion method. The antigen solutions may also contain mineral salts and one or more buffers, and also any other compound normally used in vaccines, such as stabilizers, preservatives or optionally, in addition, other adjuvants. By way of indication, the antigen concentration in the aqueous solutions is generally between 1 µg/ml and 1 mg/ml.

The method according to the invention can also include a lyophilization step. A concentrated liquid emulsion is first prepared as has just been described, but preferably choosing, as aqueous solution, water rather than a buffered solution. This emulsion is subsequently diluted in a lyophilization substrate comprising an alditol, a sugar and an alkylpolyglycoside. A lyophilization substrate normally used comprises mannitol, sucrose and dodecyl maltoside. The diluted emulsion is then divided up into samples (for example 0.5 ml) and subjected to a lyophilization cycle which can be carried out in the following way:

- loading of the samples at +4°C,
- freezing for approximately 2 hours at a set temperature of –45°C,
- primary desiccation for 14 to 19 hours at a set temperature of 0°C,
- secondary desiccation for 3 hours 30 min at a set temperature of +25°C.

The lyophilizate obtained is generally conserved at a temperature in the region of +4°C before being mixed with one or more vaccine antigens. A vaccine composition according to the invention can be thus prepared by taking up the lyophilized emulsion with an aqueous solution of antigens, and can then be conserved as it is (i.e. in the liquid state), or can be subjected to a further lyophilization cycle in order to be conserved in the form of a lyophilizate, if the nature of the antigens allows this. Alternatively, it is possible to directly dilute the concentrated emulsion with an aqueous solution comprising both the vaccine antigens and the alditol, the sugar and the alkylpolyglycoside, and to subsequently subject the composition obtained to lyophilization. Such a manner of carrying out the procedure implies, of course, that the antigens are compatible with a lyophilization process.

The examples which follow illustrate various embodiments of the invention in a nonlimiting manner.

Example I: Preparation of a concentrated thermoreversible O/W emulsion containing 32.4% of squalene (w/w)

A solution of mannitol at 18% in phosphate buffer (w/w) was prepared with mechanical stirring at 40°C. 0.093 g of Eumulgin™ B1 was added to 0.454 g of this solution, which was homogenized with mechanical stirring at 40°C for 5 min.

A stock suspension containing 1000 µg/ml of the chemical compound ER804057 in a 50 mM Tris buffer was prepared. 390 µl of the stock suspension of ER804057 were added to the ™ B1/mannitol mixture.

In another container, 0.073 g of Dehymuls™ SMO was mixed with 0.484g of squalene and the mixture was homogenized by mechanical stirring for 5 minutes at 30°C.

The content of the aqueous phase containing ER804057 was subsequently incorporated, with stirring at approximately 30°C, into the oily phase containing the Dehymuls™ SMO /squalene mixture.

The crude emulsion obtained was heated, with mechanical stirring, until the temperature reached 60°C. This temperature corresponds to the phase inversion temperature of this composition. The emulsion is then in the form of an inverse emulsion (W/O emulsion).

The heating was subsequently stopped, but the stirring was maintained until the temperature reached the ambient temperature of the laboratory (≈20°C). The emulsion returns to the form of an O/W emulsion.

A homogeneous thermoreversible O/W emulsion was thus obtained, in which more than 90% of the population by volume of the oil droplets has a size ≤ 200nm and in which the composition by weight is as follows:

32.4% of squalene,
6.2% of ceteareth-12 (Emulgin B1),
4.9% of sorbitan monooleate (dehymuls SMO),
5.5% of mannitol
0.026% of ER804057.

The amount of squalene in this adjuvant emulsion therefore represents 32.4% of the total weight of the emulsion.

In another variant, a mixture containing 50.5 g of a phosphate buffer, 6 g of mannitol, 6.18 g of Eumulgin™ B1 and 0.026 g of ER804057 was prepared in a beaker. This mixture was kept stirring at approximately 40°C. The oily phase was prepared, in another vessel, by mixing 32.5 g of squalene with 4.8 g of Dehymuls SMO, with

magnetic stirring, until the Dehymuls SMO had completely dissolved. When the homogenous phases had been obtained, the incorporation of the aqueous phase into the oily phase, and the temperature-increase steps followed by the temperature-decrease step were carried out as above. A homogenous thermoreversible O/W emulsion was obtained, in which more than 90% of the population by volume of the oil droplets have a size ≤200 nm and in which the composition by weight is as follows:

32.5% of squalene,
6.2% of ceteareth-12 (Eumulgin B1),
4.8% of sorbitan monooleate (Dehymuls SMO),
6% of mannitol,
0.026% of ER804057,
50.5% of PBS.

In another variant of the method, a citrate buffer, pH 6.04, prepared by mixing 0.83 mM of citric acid monohydrate with 9.14 mM of sodium citrate, was used in place of the PBS buffer.

This concentrated squalene emulsion was used as a "stock" emulsion, from which were derived dilute thermoreversible O/W emulsions by dilution in a phosphate buffer, a Tris buffer or a citrate buffer, which were then sterilized by filtration (see example II). These dilute O/W emulsions are subsequently mixed with one or more vaccine antigens (see examples III, IV and V).

Example II: Study of the stability of a dilute thermoreversible O/W emulsion containing 5% of squalene (w/w)

The concentrated emulsion of example 1 was diluted in a 9.6 mM phosphate buffer (pH = 7.4) so as to obtain a dilute emulsion in which the amount of squalene represents 5% of the total weight of the emulsion. The composition of the dilute emulsion, called PIT-ER804057 at 5%, was as follows:

Squalene: 50mg/ml
Ceteareth-12 (Emulgin B1): 9.5 mg/ml
Sorbitan monooleate (dehymuls SMO): 7.4 mg/ml

Mannitol: 9 mg/ml

ER804057: 40 µg/ml

The stability of this thermoreversible emulsion was evaluated after storage for 6 months at a temperature of +4°C by verifying the ER804057 content in the emulsion and the size distribution of the emulsion. To assay ER804057, a selective extraction of ER804057 from the emulsion was carried out, followed by analysis by high performance liquid chromatography (HPLC) coupled to a diode array detector (UV detection). The ER804057 content of the emulsion to be verified was determined using a standard range containing between 5 and 25 µg/ml of ER804057. In order to compensate for the variations in extraction yields, a constant amount of an internal standard, the chemical structure of which is very similar to that of ER804057, was introduced into each sample to be assayed (including into the samples of the standard range). This internal standard is the chemical molecule called ER803022.

The standard range was prepared from a thermoreversible emulsion which has the same composition and has been prepared in the same way as the PIT-ER804057 at 5% emulsion (cf. example II), except that it did not contain any ER804057 (emulsion PIT at 5%), to which were added a varying amount of ER804057 taken from a stock solution of ER804057 at 0.1 mg/ml of a mixture containing 2 volumes of chloroform per volume of methanol (mixture CM 2:1), and a fixed amount of an internal standard (10 µg), taken from a stock solution of an internal standard at 0.1 mg/ml of mixture CM 2:1, which was diluted as appropriate in water for injectable preparation.

The sample of PIT-ER804057 at 5% to be assayed was prepared by taking an aliquot of the emulsion PIT-ER804057 at 5%, to which 10 µg of internal standard were added, and which was diluted in water for injectable preparation.

The extraction of ER804057 from the samples of the standard range or from the samples of PIT-ER804057 at 5% was carried out in the following way: The sample was solubilized with CM 2:1. The two-phase system obtained is composed of a chloroform phase containing predominantly ER804057 and an aqueous phase containing the other compounds of the emulsion. The chloroform phase was recovered and evaporated under hot conditions under a stream of nitrogen. The dry extract obtained was taken up and again solubilized in the CM 2:1 mixture. The mixture was loaded onto an anion

exchange cartridge pre-equilibrated in the CM 2:1 mixture. It selectively retained ER804057 and the internal standard which are negatively charged, whereas the other components of the emulsion, which were not charged, were eliminated. ER804057 and the internal standard were eluted by means of a mixture containing 2 volumes of chloroform and 3 volumes of methanol, per volume of 1M NaCl. The eluate was subsequently dried under hot conditions under a stream of nitrogen. Finally, a final extraction was carried out with water and CM 2:1 so as to eliminate the residual salts and recover ER804057 and the internal standard in the chloroform phase, which was finally evaporated under hot conditions under a stream of nitrogen. The dry extract derived from each sample was conserved at -20°C before being analyzed by HPLC.

The dry extract of each sample was taken up with 50 µl of CM 4:1, and then diluted to 1/2 in methanol, then to 1/10th in a mixture of 30% acetonitrile-water for injectable preparation. 20 µl of the dilution were injected into a liquid chromatography apparatus (Merck Hitachi HPLC, Lachrom series 7000) comprising a Waters XTerra™ RP8 column pre-equilibrated in a mobile phase consisting of 80% of phase A (50/50 water for injectable preparation/ethanol containing 2% of H_3PO_4) and 20% of phase B (ethanol containing 2% of H_3PO_4). ER804057 and the internal standard were eluted using a gradient of ethanol containing 2% of H_3PO_4. At the outlet of the HPLC, the eluate reached the diode array detector and the molecules were detected at the wavelength of 215 nm. On the chromatogram obtained, the surface areas of the 2 peaks (analyte and reference) were integrated and correlated. In order to correct the variations related to the preparation of the sample, the standard curve was established between the ratio of the surface areas of the peaks corresponding to the ER 804057 (quantified molecule) and ER803022 (internal standard) couple and the ratio of the concentrations corresponding to ER804057 and ER803022 (internal standard). Once the curve was established, the amount of ER804057 present in the emulsion PIT-ER804057 at 5% was determined by measuring the ratio of the surface areas of the ER804057/internal standard peaks and comparing with the standard curve.

	1 month at +4°C	3 months at +4°C	6 months at +4°C
ER 804057 (theoretical concentration 40 µg/ml)	38 µg/ml	37 µg/ml	42 µg/ml

The results mentioned in the table above show that ER804057 conserves its structural integrity and that its concentration in the emulsion PIT-ER804057 at 5% is not substantially varied after the emulsion has been conserved for 6 months at + 4°C.

The size distribution analyses were carried out, after dilution of the emulsion to 1/100, with the Mastersizer 2000, using the following parameters: IR particle = 1.495; IR medium = 1.332; absorption value = 0; lower obscuration limit = 4%; upper obscuration limit = 7%; "general purpose" analysis model. For each size distribution analysis, the following parameters were evaluated: d10, d50 and d90, which represent respectively the values of the mean particle diameters below which 10%, 50% and 90%, respectively, of the population by volume of oil droplets is found.

	T = 0	T = 3 months	T = 6 months
d10	71 nm	70 nm	70 nm
d50	103 nm	100 nm	101 nm
d90	155 nm	152 nm	153 nm

These results show that the size distribution of the emulsion is stable at +4°C over a time period of at least 6 months.

Example III: Vaccine composition against cytomegalovirus infections, prepared from an O/W emulsion according to the invention

Vaccine compositions comprising, as vaccine antigen, a recombinant protein which derives from the gB glycoprotein of CMV were prepared. This recombinant protein was produced by a recombinant CHO line transfected with a plasmid called pPRgB27clv4, which contains a modified gB gene. To facilitate the production of this recombinant

protein by the CHO line, the gB gene, the sequence of which is described in US 5,834,307, was modified beforehand by deleting the part of the gene which encodes the transmembrane region of the gB protein corresponding to the amino acid sequence between valine 677 and arginine 752 and introducing 3 point mutations at the cleavage site. The protein produced by the CHO line, called gBdTM, corresponds to a truncated gB protein depleted of the cleavage site and of the transmembrane region.

The construction of the plasmid pPRgB27clv4 and the production of the truncated gB protein (gBdTM) by the recombinant CHO line are described in US 6,100,064. The gBdTM protein produced in the culture medium is subsequently purified by affinity chromatography using the monoclonal antibody 15D8 described by Rasmussen L et al. (J. Virol. (1985) 55: 274-280). The purified protein was stored in the form of a stock solution containing 0.975 mg/ml of gBdTM in phosphate buffer.

Immunostimulating compositions of gBdTM and formulated with various compositions of O/W emulsions or with a suspension of aluminum hydroxide were prepared.

Composition No. 1 contained 2 µg of gBdTM in citrate buffer at pH 6 in 50 µl (group gB).

Composition No. 2 contained 2 µg of gBdTM, 1.075 mg of squalene, 0.133 mg of sorbitan trioleate (Montane™ VG 85) and 0.125 mg of Tween™80 in citrate buffer at pH 6 in 50 µl (group gB+O/W emulsion). This composition was obtained by mixing, volume for volume, a solution of gB with an O/W emulsion of the prior art which was obtained by microfluidization.

Composition No. 3 contained 2 µg of gBdTM and 60 µg of aluminum hydroxide in phosphate buffer in 50 µl (group gB+AL).

Composition No. 4 contained 2 µg of gB, 1.25 mg of squalene, 0.187 mg of Dehymuls™ SMO, 0.237 mg of Eumulgin™ B1 and 0.225 mg of mannitol in PBS buffer at pH 7.4 in 50 µl. This composition was obtained by mixing, volume for volume, a solution of gB with a thermoreversible O/W emulsion containing 5% of squalene (group gB+PIT). The thermoreversible O/W emulsion used to prepare this composition was obtained by dilution of a concentrated thermoreversible O/W emulsion containing 32.5% of squalene (w/w), which has been prepared using the same method as that described in example 1,

except for the fact that the aqueous phase did not contain any ER804057.

Composition No. 5 contained 2 µg of gBdTM and 1 µg of ER804057, in a citrate buffer, pH 6, in 50 µl (group gB+ER804057).

Composition No. 6 contained 2 µg of gBdTM, 1.25 mg of squalene, 0.145 mg of Montane™ VG 85, 0.147 mg of Tween™80 and 1 µg of ER804057 in citrate buffer at pH 6 in 50 µl (group gB+O/W emulsion+ER804057). This composition was obtained by mixing, volume for volume, a solution of gB with an O/W emulsion of the prior art obtained by microfluidization, to which ER804057 has been added.

Composition No. 7 contained 2 µg of gBdTM, 1 µg of ER804057 and 60 µg of aluminum hydroxide in a phosphate buffer in 50 µl (group gB+Al+ER804057).

Composition No. 8 contained 2 µg of gB, 1.25 mg of squalene, 0.189 mg of Dehymuls™ SMO, 0.240 mg of Eumulgin™ B1, 0.211 mg of mannitol and 1 µg of ER804057 in PBS buffer at pH 7.4 in 50 µl. This composition was obtained by mixing, volume for volume, a solution of gB with a thermoreversible O/W emulsion PIT-ER804057 at 5% of squalene, obtained by dilution of the stock emulsion of example 1 (group gB+PIT+ER804057).

Eight groups of ten 8-week-old female outbred OF1 mice were immunized subcutaneously, on days D0 and D21, with the compositions indicated above (each group of mice was given 2 injections of the same composition).

Blood samples were taken from the retro-orbital sinus on D20 and on D34 and were used to determine the gBdTM-specific IgG1 and IgG2a antibody concentrations. These assays were carried out by ELISA, by coating the wells of Dynex 96-well microplates with 100 ng (100 µl) of gBdTM in 0.05 M carbonate buffer solution at pH 9.6, at +4°C overnight.

To determine the neutralizing antibodies, the protocol described by Gonczol E. et al. in J. Virological Methods, 14: 37-41 (1986) was used.

MRC5 cells cultured in an MEM medium containing 10% fetal calf serum were used, between passages 28 and 38, for the microneutralization analyses. The Towne CMV strain (Wistar Institute, Philadelphia, US) purified and propagated on MRC5 cells, having a titer of approximately 2 x 10^6 PFU/ml was used as infection strain. A source of complement obtained from the sera of mice from the Virion Ltd Institute (Switzerland)

was also used. A mixture of human sera having a titer at 1:128 was used as a positive control, and is included in each microneutralization assay.

The sera to be tested were inactivated by heating at 56°C for 30 minutes. 105 µl of culture medium (MEM + 10% fetal calf serum) were added to a 15 µl aliquot of each inactivated serum, in flat-bottomed 96-well culture plates (1/8 dilution). Serial 2-fold dilutions were then prepared. The control sera were tested in the same way. 60 µl of virus suspension containing 3000 PFU and 5 µl of mouse complement were added to each well. After incubation for 1 hour at 37°C under CO_2, 3-4 x 10^4 MRC5 cells in a volume of 150 µl of culture medium were added to each of the wells. The microcultures were cultured for 4 days. The cytopathic activity of the virus was 100% in the wells which did not contain any sera. On the other hand, an inhibition of the cytopathic activity of the virus was observed in the wells which contained neutralizing sera. The neutralizing antibody titer of a serum corresponds to the inverse of the dilution thereof which inhibits the cytopathic activity of the virus by more than 90%.

The results which were obtained for each group of mice are given in the tables hereinafter:

Group of mice	IgG1 at D20	IgG2a at D20	IgG1 at D34	IgG2a at D34	ratio at D34 IgG1/IgG2a
Group gB	2.47*	2.09	3.80	2.94	137
Group gB+O/W emulsion	4.06	2.98	5.49	4.18	143
Group gB+AL	3.06	1.85	4.90	3.33	357
Group gB+PIT	4.61	3.91	5.61	4.85	14
Group gB+ER804057	3.09	3.12	4.43	4.16	6
Group gB+PIT+ER804057	4.78	4.58	5.83	5.74	3

*: mean titer of the dilutions of sera (expressed as $\log_{10}$)

These results show that the PIT-ER804057 emulsion has a greater immunostimulating capacity than the other adjuvants since the specific IgG1 and IgG2a titers obtained in the " gB+PIT+ ER804057" group of mice are significantly higher than those obtained in the " gB+AL" or "gB+O/W emulsion" groups of mice. The immunostimulating capacity of

the emulsion according to the invention is not due only to the thermoreversible emulsion (PIT emulsion) or to the TLA4 agonist, but to the combination of the two products. The specific IgG1 and IgG2a titers observed in the " gB+PIT" and " gB+ER804057 " groups are in fact significantly lower than those which are observed in the " gB+PIT+ ER804057" group.

Summarizing table of neutralizing antibody production

Group of mice	Mean neutralizing antibody titer
Group gB	16**
Group gB+O/W emulsion	32
Group gB+AL	32
Group gB+PIT	48
Group gB+ER804057	16
Group gB+O/W emulsion H/E+ER804057	32
Group gB+AL+ER804057	32
Group gB+PIT+ER804057	128

**: inverse of the mean of the serum dilutions which inhibit the cytopathic effect of the virus by more than 90%.

These results show that the immunostimulating composition resulting from the mixing of a CMV envelope antigen with a thermoreversible O/W emulsion containing a TLR4 agonist as described in the invention is that which induces the highest neutralizing antibody titer in the mice. The PIT-ER804057 emulsion has a greater capacity for stimulating the production of neutralizing antibodies than the other adjuvant compositions tested. The PIT-ER804057 emulsion is found to be more effective (in terms of its capacity for stimulating neutralizing antibody production) than a squalene-based O/W emulsion of the prior art containing the same components as the MF59 emulsion, considered up until now to be the reference adjuvant for adjuventing CMV proteins. It is also noted that the addition of a TLR4 agonist to the O/W emulsion of the prior art does not increase the effectiveness of this emulsion (the neutralizing antibody titer remains the same), whereas the effectiveness of a thermoreversible emulsion (PIT)

increases when it contains a TLR4 agonist (the neutralizing antibody titer increases).

Example IV: Vaccine composition against the flu prepared from an O/W emulsion according to the invention

Immunostimulating compositions were prepared from an anti-flu vaccine composition comprising the 3 vaccine strains of the 2004 campaign (the A/New Caledonia (H1N1) strain, the A/Wyoming (H3N2) strain, and the B/Jiangsu strain, which is formulated with various compositions of O/W emulsions or with a suspension of aluminum hydroxide.

Composition No. 1 contained 0.3 µg of hemagglutinin (HA) of each of the viral strains in PBS buffer in 30 µl. (0.3 µg HA group).

Composition No. 2 contained 6.3 µg of hemagglutinin (HA) of each of the viral strains in PBS in 30 µl. (6.3 µg HA group).

Composition No. 3 contained 0.3 µg of hemagglutinin (HA) of each of the viral strains, 0.65 mg of squalene, 0.075 mg of sorbitan trioleate (Span™ 85) and 0.075 mg of Tween™80 in PBS buffer in 30 µl (0.3 µg HA+O/W emulsion group). This composition was obtained by mixing the anti-flu vaccine composition with an O/W emulsion of the prior art obtained by microfluidization.

Composition No. 4 contained 0.3 µg of hemagglutinin (HA) of each of the viral strains, 0.75 mg of squalene, 0.11 mg of Dehymuls™ SMO, 0.143 mg of Eumulgin™ B1 and 0.138 mg of mannitol and 0.6 µg of ER804057 in PBS buffer at pH 7.4 in 30 µl (0.3 µg HA+PIT+ER804057 group). This composition was obtained by mixing the anti-flu vaccine composition with the thermoreversible emulsion as described in example 1 and which has been diluted beforehand in PBS buffer.

Four groups of eight 8-week-old female BALB/c mice were immunized by administering intradermally (inner face of the ear), on D0, a dose of 30 µl of one of the immunostimulating compositions indicated above.

Blood samples were taken from the retro-orbital sinus on D21 and were used to determine the titers of neutralizing antibodies specific for each viral strain (hemagglutination-inhibiting (HAI) antibodies) obtained in each group of immunized mice. The principle of this assay is based on the ability of flu viruses to agglutinate red

blood cells, whereas a serum which contains neutralizing antibodies directed specifically against the HA of the virus inhibits the "hemagglutinating" activity of the virus. Firstly, the nonspecific inhibitors contained in the serum were eliminated by treating the latter with an RDE enzyme (receptor destroying enzyme) provided by Sigma and then bringing them into contact with a 10% solution of chicken red blood cells. A supernatant freed of nonspecific inhibitors and which corresponded to a serum diluted to 1/10th was obtained. Serial 2-fold dilutions of the supernatant in phosphate buffer were subsequently prepared and then 50 µl of each of the dilutions were deposited into the wells of a V-bottomed microplate. 50 µl of a viral suspension originating from a clarified allantoic fluid titered at 4 hemagglutinating units (4HAU) were added to each well. The plate was incubated for 1 hour at laboratory temperature before the addition of 50 µl of a solution of chicken or turkey red blood cells to each of the wells. The plate was left to stand for 1 hour at +4°C before the assay was read. The presence of hemagglutination inhibition was reflected by the presence of a red spot at the bottom of the microwell, whereas the presence of hemagglutination was reflected by the presence of a pinkish halo in the microwell. The HAI antibody titer is represented by the inverse of the final dilution at which no hemagglutination is observed in the microwell.

The results that were obtained are given in the table below:

Group of mice	HAI against A /New Caledonia (H1N1)	HAI against A/Wyoming (H3N2)	HAI against B/Jiangsu
0.3 µg HA	26***	174	8
6.3 µg HA	247	907	73
0.3µg HA+O/W emulsion	135	987	57
0.3µg HA+PIT+ER804057	290	1522	98

***: mean of the HAI titers obtained on the 8 sera of each group of mice.

These results show that the vaccine composition obtained by mixing a flu vaccine with a thermoreversible O/W emulsion containing a TLR4 agonist is that which induces the highest neutralizing antibody titer in the mouse irrespective of the vaccine strain tested,

compared with the other vaccine compositions. The PIT-ER804057 emulsion is found to be even slightly more effective (in terms of its capacity for stimulating neutralizing antibody production) than an O/W emulsion of the prior art, the composition of which is similar to MF59. The advantage of this emulsion also lies in the fact that the amount of antigen can be greatly reduced since the results obtained with a dose of 0.3 μg of hemagglutinin mixed with a PIT-ER804057 emulsion are better than those which are obtained with a dose of hemagglutinin that is 20 times higher.

In another assay, the evolution of the hemagglutinin-inhibiting antibody titer over time was followed in groups of mice immunized with various immunostimulating compositions prepared from the same vaccine from the 2004 campaign.

Composition No. 1 contained 0.3 μg of hemagglutinin (HA) of each of the viral strains in PBS buffer in 30 μl (0.3 μg HA group).
Composition No. 2 contained 6.3 μg of hemagglutinin (HA) of each of the viral strains in PBS buffer in 30 μl (6.3 μg HA group).
Composition No. 3 contained 0.3 μg of hemagglutinin (HA) of each of the viral strains and 0.6 μg of ER804057 in an aqueous buffer in 30 μl (0.3 μg HA+ER804057 group).
Composition No. 4 contained 0.3 μg of hemagglutinin (HA) of each of the viral strains, 0.30 mg of squalene, 0.044 mg of Dehymuls™ SMO, 0.057 mg of Eumulgin™ B1 and 0.055 mg of mannitol in PBS buffer at pH 7.4 in 30 μl (0.3 μg HA+PIT at 1% group).
Composition No. 5 contained 0.3 μg of hemagglutinin (HA) of each of the viral strains, 0.30 mg of squalene, 0.044 mg of Dehymuls™ SMO, 0.057 mg of Eumulgin™ B1, 0.055 mg of mannitol and 0.6 μg of ER804057 in PBS buffer at pH 7.4 in 30 μl (0.3 μg HA+PIT at 1%+ER804057 group).

Five groups of five 8-week-old female BALB/c mice were immunized by administering intradermally (inner face of the ear), on D0, a dose of 30 μl of one of the immunostimulating compositions indicated above.
Blood samples were taken from the retro-orbital sinus on D23, D51 and D79 and were used to determine the titers of neutralizing antibodies specific for the H1N1 strain (hemagglutination-inhibiting (HAI) antibodies) obtained in each group of immunized

mice. The results that were obtained are given in the table below.

Group of mice	D23	D51	D79
0.3 µg HA group	35***	53	80
6.3 µg HA group	235	243	279
0.3 µg HA+ER804057 group	65	211	243
0.3 µg HA+PIT at 1% group	226	557	735
0.3 µg HA+PIT at 1%+ER804057 group	226	970	844

***: mean of the HAI titers obtained on the 5 sera of each group of mice.

The effectiveness of the PIT-ER804057 emulsion in terms of its ability to produce flu virus hemagglutination-inhibiting antibodies (protective antibodies) is the result of the combined action of the emulsion and of the TLR4 agonist; the PIT emulsion alone or ER804057 alone is less effective.

Example V: Vaccine composition against the flu, prepared from an emulsion according to the invention tested in a population of young or old mice already sensitized to the flu virus

The effectiveness of the emulsion of the invention was tested in the case of a vaccine against the flu which would be administered to individuals already sensitized to the flu virus, either because these individuals have already been in contact with the flu virus or because they have already been vaccinated with a flu vaccine.

To carry out this type of evaluation, mice preimmunized intramuscularly (IM) with a trivalent vaccine can be used, according to Potter et al. (Vaccine, 2003, 21:940-945), as an animal model that is not naïve with respect to the flu.

5 groups of 10 C57Bl/6 mice 8-10 weeks old received by IM injection, a dose of trivalent vaccine containing 1.5 µg of HA of each of the A/New Caledonia/20/99 (H1N1), A/New York/55/04 (H3N2) and B/Malaysia/2506/04 strains.

At D28, with the exception of one group (PBS group) which received an injection of a PBS buffer, all the other groups of mice received, intradermally, in a volume of 30 µl, various vaccine compositions containing a trivalent vaccine different than that which was used for the primary immunization (A/New Caledonia/20/99 (H1N1), A/Wellington/01/04 (H3N2) and B/Jiangsu/10/03).

One group received a composition containing 0.3 µg of HA of each of the strains in PBS buffer (0.3 µg HA group).

Another group received a composition containing 6.3 µg of HA of each of the strains in PBS buffer (6.3 µg HA group).

Another group received a composition containing 0.3 µg of HA of each of the strains in PBS buffer in an O/W emulsion at 1% of squalene containing 0.3 mg of squalene, 0.044 mg of Dehymuls™ SMO, 0.057 mg of Eumulgin™ B1 and 0.055 mg of mannitol in PBS buffer. This composition which contained 1% of squalene was prepared by mixing the flu vaccine with an O/W emulsion obtained by diluting a concentrated thermoreversible stock solution prepared according to the same method as that described in example 1, except for the fact that the aqueous phase did not contain any ER804057 (0.3 µg HA +PIT 1% group).

Finally, the last group received a composition containing 0.3 µg of HA of each of the strains in PBS buffer in an O/W emulsion at 1% of squalene containing 0.3 mg of squalene, 0.44 mg of Dehymuls™ SMO, 0.057 mg of Eumulgin™ B1 and 0.055 mg of mannitol in PBS buffer and 0.6 µg of ER804057. This composition which contained 1% of squalene and 0.6 µg of ER804057 was prepared by mixing the flu vaccine with an O/W emulsion obtained by diluting a concentrated thermoreversible stock solution prepared according to the same method as that described in example 1 (0.3 µg HA+PIT 1%/ER 804057 group).

At D50, the mice were sacrificed by euthanasia in order to collect a blood sample and to take a spleen sample.

An assay of the HAIs with respect to the A/New Caldedonia/20/99 (H1N1), A/Wellington/01/04 (H3N2) and B/Jiangsu/10/03 strains was carried out on each blood sample. The results that were obtained are given in the table below:

Group of mice	HAI against A/New Caledonia (H1N1)	HAI against A/Wellington (H3N2)	HAI against B/Jiangsu
PBS group	52*	48	13
0.3 µg HA group	95	226	57
6.3 µg HA group	190	640	226
0.3 µg HA +PIT 1% group	431	640	290
0.3 µg HA+PIT 1%/ER804057 group	698	2348	640

* represents the mean value of the HAI titers obtained on the 10 sera from each group of mice

These results show that the average HAI titer in the group of mice immunized with the "HA +PIT 1%/ER804057" vaccine composition containing a low dose of squalene (1%) and of TLR4 agonist (it contains 0.6 µg of ER804057) is significantly higher than the titers obtained after immunization of the mice with the nonadjuvanted flu vaccine at an equivalent dose of HA (0.3 µg HA group) or at a dose of HA 20 times higher (6.3 µg HA group). These results show the advantage of the PIT 1%/ER 804057 emulsion, even within a population already sensitized to the flu virus, since the amount of flu antigen of each strain can be reduced by a factor of 20, while at the same time obtaining higher protective antibody titers with respect to the 3 strains of virus.

The specific cellular immune response was analyzed on each spleen sample using the ELISPOT technique and the CBA (Cytometric Bead Array) technique to assay the interferon-γ and the IL5 produced by the splenocytes after specific stimulation.

As regards the ELISPOT technique, 2×10^5 splenocytes in 200 µl of a culture medium (RPMI 1640, 10% of fetal calf serum, 2 mM glutamine, 50 mM β-mercaptoethanol) were deposited in the wells of nitrocellulose microplates presensitized with a rat anti-mouse IFNγ antibody (Pharmingen ref: 551216) or with a rat anti-mouse IL5 antibody (Pharmingen ref: 554393). The splenocytes were incubated overnight at 37°C in the

presence of murine IL-2 (Bohringer) (10 U/ml) and of various flu antigens at a concentration of 1 μg/ml. For the analysis of the CD8+ cell response, a flu NP peptide (TYQRTRALV) recognized in the H-2kd context was used. For the analysis of the CD4+ cell response, the flu antigen is represented by the trivalent inactivated split vaccine containing the A/New Caledonia/20/99 (H1N1), A/Wellington/01/04 (H3N2) and B/Jiangsu/10/03 strains. The microplates were then washed and the splenocytes which secreted IFNγ or IL5 were detected by means of a biotinylated rat anti-mouse IFNγ antibody (Pharmingen ref: 554410) or a biotinylated rat anti-mouse IL5 antibody (Pharmingen ref: 554393) and by means of peroxidase-conjugated streptavidin (Southern Biotechnology-ref 7100-05). After revelation using 3-amino-9-ethylcarbazol, the spots corresponding to the splenocytes which secrete IFNγ or IL5 were counted by means of an automatic ELISPOT reader. The results were expressed as number of cells secreting IFNγ or IL5 per 10^6 splenocytes. The positive detection threshold is 20 spots by 10^6 splenocytes.

As regards the CBA technique, 4×10^5 splenocytes in 200 μl of a culture medium (RPMI 1640, 10% of fetal calf serum, 2 mM glutamine, 50 mM β mercaptoethanol) were deposited in the wells of culture microplates. The splenocytes were incubated for 5 days at 37°C in the presence of the trivalent vaccine (at 1 μg/ml) or in the absence of stimulating agent in order to evaluate the nonspecific production of cytokines (medium control). The IFNγ content or the IL5 content of the culture supernatants was then assayed by flow cytometry using the mouse Th1/Th2 CBA kit (Becton Dickinson - ref: 551287). The positive detection threshold was 2.5 pg/ml for IFNγ and 5 pg/ml for IL5. For each spleen sample, the specific concentration of IFNγ or of IL5 was calculated by subtracting from the result the amount of IFNγ or IL5 which is produced nonspecifically.

The results of the cellular response analyses are given in the table below:

Group of mice	Number of splenocytes secreting IFNγ	Number of splenocytes secreting IL5	Amount of IFNγ in the culture supernatant	Amount of IL5 in the culture supernatant	IFNγ/IL5 ratio
PBS	19*	23*	148**	355**	2.1***
0.3 µg HA	17	66	295	400	1.3
6.3 µg HA	30	13	780	523	
0.3 µg HA+PIT 1%	31	74	691	1301	0.6
0.3 µg HA+PIT 1%/ER 804057	66	22	1499	640	4.1

* represents the mean value of the number of splenocytes secreting IL5 or IFNγ per 10^6 splenocytes after stimulation with the trivalent vaccine. The mean value is calculated on the basis of the ELISPOT results obtained on the 10 spleen samples/group of mice.

** represents the mean amount (pg/ml) of IL5 or IFNγ calculated on the basis of the results obtained on the 10 spleen samples/group of mice using the CBA technique.

*** the ratio represents the arithmetic mean of the IFNγ/IL5 ratios in each group. The IFNγ/IL5 ratio was determined for each sample on the basis of the values of the specific concentrations of IFNγ and of IL5 obtained according to the CBA method after the splenocytes had been cultured, and then the arithmetic mean of the 10 ratios was calculated for each group of mice.

These results show that the PIT 1%/ER804057 emulsion strongly orients the CD4+ cellular response toward the production of IFNγ subsequent to specific restimulation of the cells with the flu vaccine. The most pronounced Th1 response (highest IFNγ/IL5 ratio) was observed in the group of mice which received this emulsion. The Th1 response is in fact stronger than that which is observed in the group of mice which received a flu vaccine containing a 20-times higher dose (6.3 µg group). This emulsion is therefore recommended in populations of individuals who have a poor Th1 response subsequent to a flu vaccination, in particular in elderly individuals.

2007204295 22 Oct 2012

The same experimental protocol was reproduced using 17-month-old male C57B16 mice, and then the titer of HAIs directed against A/Wellington (H3N2) was measured. The results that were obtained are given in the table below:

Group of mice	HAIs against A/Wellington (H3N2)
6.3 µg HA group	104*
0.3 µg HA+PIT 1% group	247
0.3 µg HA+PIT 1%/ER804057 group	476

* represents the mean value of the HAI titers obtained on the 10 sera from each group of mice

These results show the advantage of a vaccine composition which was obtained by mixing a flu vaccine in an O/W emulsion containing a low dose of squalene (1%) and of TLR4 agonist (ER804057 at 0.6 µg) in order to vaccinate a population of elderly individuals already sensitized to the flu virus, since the amount of flu antigen of each strain can be reduced by a factor of 20, while at the same time having higher protective antibody titers with respect to the 3 strains of virus.

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.

2007204295 22 Oct 2012

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. An Oil-in-Water (O/W) emulsion comprising:

i) a TLR4 agonist, called TLA4, in which TLA4 is a chemical compound of formula I, II, III or IV,

ii) squalene,

iii) an aqueous solvent,

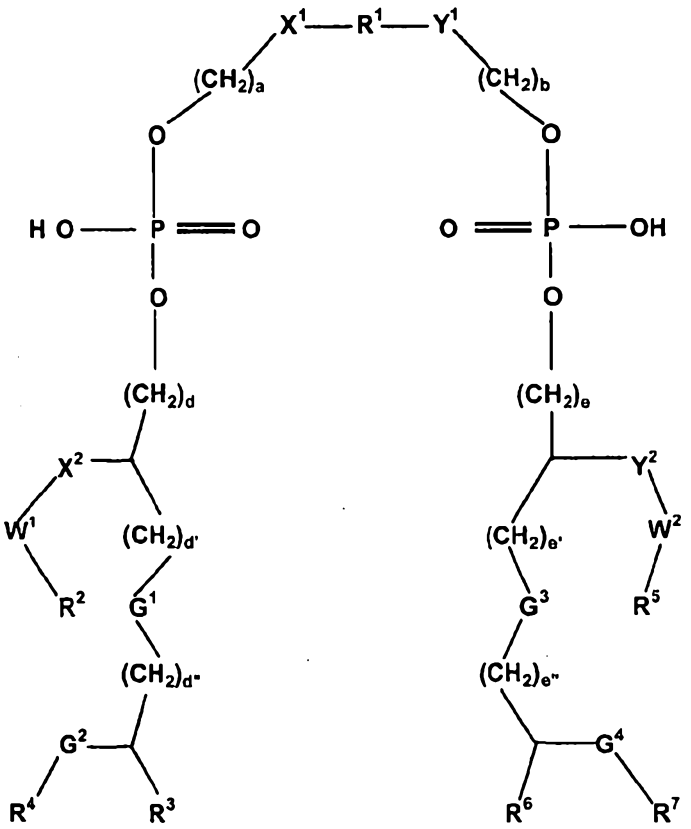
iv) a nonionic hydrophilic surfactant which is a polyoxyethylene alkyl ether,

v) a nonionic hydrophobic surfactant, and

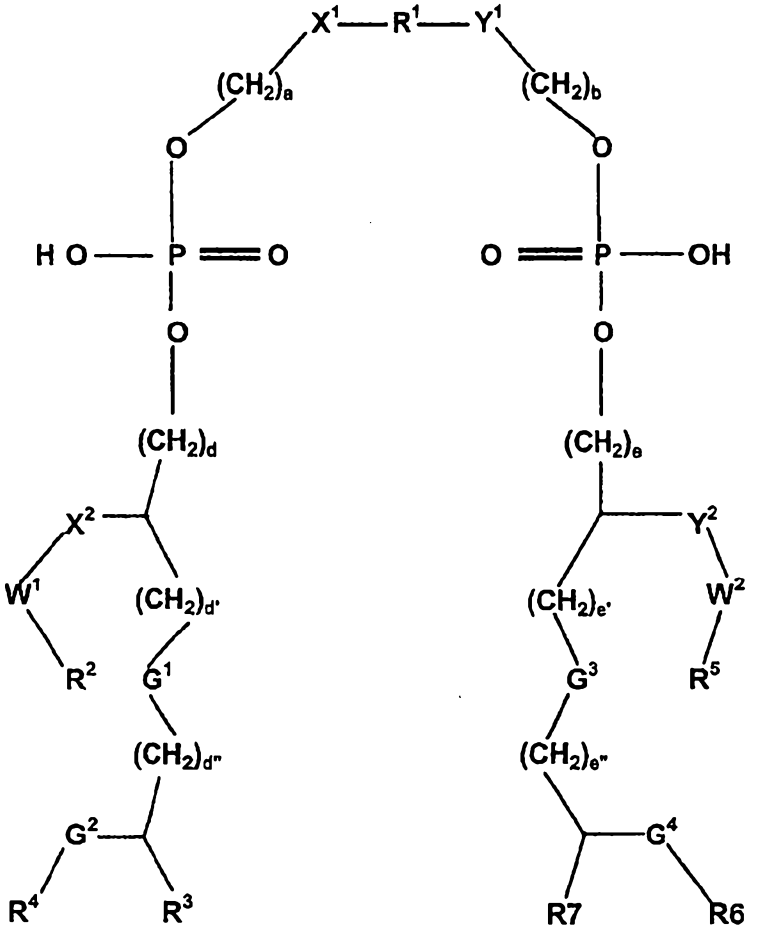
which is thermoreversible;

wherein formula I, II, III and IV are as follows:

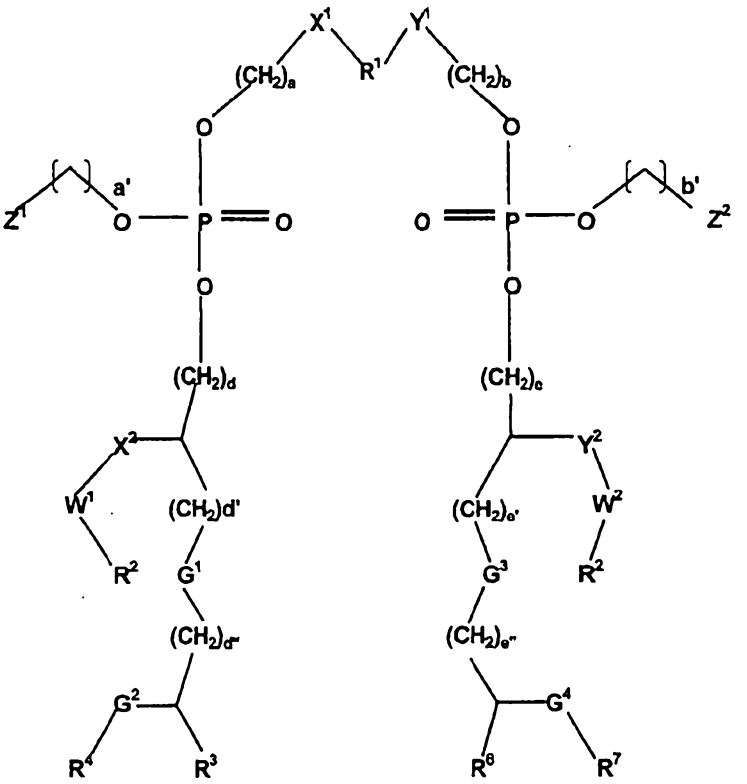
Compound of formula I



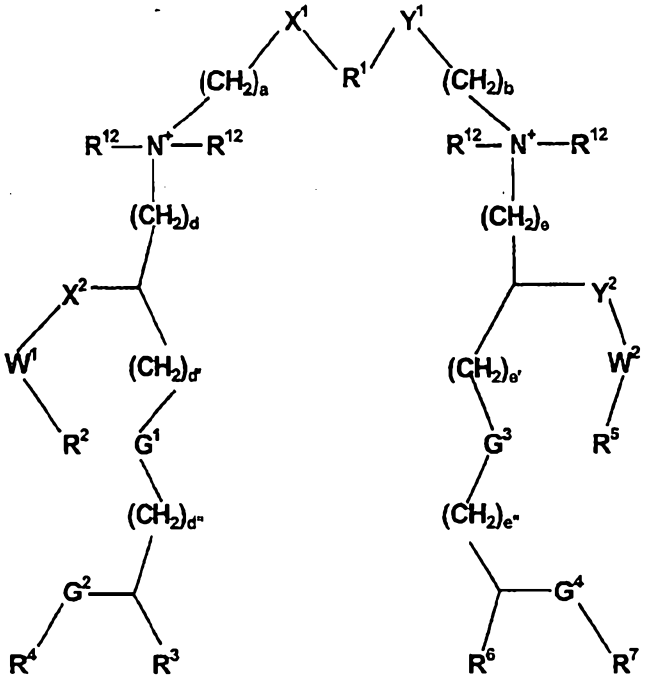
Compound of formula II



Compound of formula III



Compound of formula IV



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in which, for each of formula I, II, III or IV, R^1 is selected from the group consisting of:

a) C(O);

b) C(O)-(C_1-C_{14} alkyl)-C(O), in which said C_1-C_{14} alkyl is optionally substituted with a hydroxyl, a C_1-C_5 alkoxy, a C_1-C_5 alkylenedioxy, a (C_1-C_5 alkyl)amino or a (C_1-C_5 alkyl)aryl, in which said aryl moiety of said (C_1-C_5 alkyl)aryl is optionally substituted with a C_1-C_5 alkoxy, a (C_1-C_5 alkyl)amino, a (C_1-C_5 alkoxy)amino, a (C_1-C_5 alkyl)amino(C_1-C_5 alkoxy), -O-(C_1-C_5 alkyl)amino (C_1-C_5 alkoxy), -O-(C_1-C_5 alkyl)amino-C(O)-(C_1-C_5 alkyl)-C(O)OH, or -O-(C_1-C_5 alkyl)amino-C(O)-(C_1-C_5 alkyl)-C(O)-(C_1-C_5)alkyl;

c) an alkyl comprising a C_2-C_{15} linear or branched chain, optionally substituted with a hydroxyl or an alkoxy; and

d) -C(O)-(C_6-C_{12} arylene)-C(O)- in which said arylene is optionally substituted with a hydroxyl, a halogen, a nitro or an amino;

a and b are independently 0, 1, 2, 3 or 4;

d, d', d", e, e' and e" are independently 0, 1, 2, 3 or 4;

X^1, X^2, Y^1 and Y^2 are independently selected from the group consisting of null, an oxygen, NH and N (C(O)(C_1-C_4 alkyl)), and N(C_1-C_4 alkyl);

W^1 and W^2 are independently selected from the group consisting of a carbonyl, a methylene, a sulfone and a sulfoxide;

R^2 and R^5 are independently selected from the group consisting of:

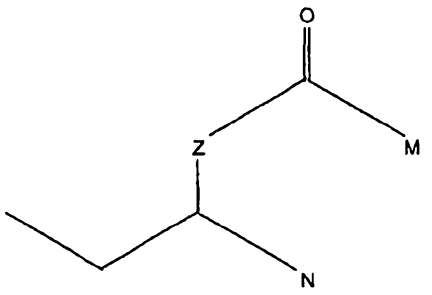
a) a C_2 to C_{20} straight chain or branched chain alkyl, which is optionally substituted with an oxo, a hydroxyl or an alkoxy;

b) a C_2 to C_{20} straight chain or branched chain alkenyl or dialkenyl, which is optionally substituted with an oxo, a hydroxyl or an alkoxy;

c) a C_2 to C_{20} straight chain or branched chain alkoxy, which is optionally substituted with an oxo, a hydroxyl or an alkoxy;

d) NH-(C_2 to C_{20} straight chain or branched chain alkyl), in which said alkyl group is optionally substituted with an oxo, a hydroxyl or an alkoxy; and

e)



in which Z is selected from the group consisting of O and NH, and M and N are independently selected from the group consisting of an alkyl, an alkenyl, an alkoxy, an acyloxy, an alkylamino and an acylamino comprising a C_2-C_{20} linear or branched chain;

R^3 and R^6 are independently selected from the group consisting of a C_2 to C_{20} straight chain or branched chain alkyl and alkenyl, optionally substituted with an oxo or a fluoro;

R^4 and R^7 are independently selected from the group consisting of C(O)-(C_2 to C_{20} straight chain or branched chain alkyl or alkenyl), a C_2 to C_{20} straight chain or branched chain alkyl, a C_2 to C_{20} straight chain or branched chain alkoxy, and a C_2 to C_{20} straight chain or branched chain alkenyl; in which said alkyl, alkenyl or alkoxy groups can be independently and optionally substituted with a hydroxyl, a fluoro or a C_1-C_5 alkoxy;

G_1, G_2, G_3 and G_4 are independently selected from the group consisting of an oxygen, a methylene, an amino, a thiol, -C(O)NH-, -NHC(O)-, and -N(C(O)(C_1-C_4 alkyl))-;

or G^2R^4 or G^4R^7 can together be a hydrogen atom or a hydroxyl;

and in which, for formula III:

a' and b' are independently 2, 3, 4, 5, 6, 7 or 8, preferably 2;

Z^1 is selected from the group consisting of $-OP(O)(OH)_2$, $-P(O)(OH)_2$, $-OP(O)(OR^8)(OH)$ where R^8 is a C_1-C_4 alkyl chain, $-OS(O)_2OH$, $-S(O)_2OH$, $-CO_2H$, $-OB(OH)_2$, -OH, $-CH_3$, $-NH_2$ and $-NR^9{}_3$ where R^9 is a C_1-C_4 alkyl chain;

Z^2 is selected from the group consisting of $-OP(O)(OH)_2$, $-P(O)(OH)_2$, $-OP(O)(OR^{10})(OH)$ where R^{10} is a C_1-C_4 alkyl chain, $-OS(O)_2OH$, $-S(O)_2OH$, $-CO_2H$,

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2007204295 22 Oct 2012

$-OB(OH)_2$, -OH, $-CH_3$, $-NH_2$ and $-NR^{11}$ where R^{11} is a C_1-C_4 alkyl chain;

and in which, for formula IV:
R12 is H or a C_1-C_4 alkyl chain;

or a pharmaceutically acceptable salt of the compound of chemical formula I, II, III or IV.

2. The emulsion as claimed in claim 1, in which at least 90% of the population by volume of the oil droplets has a size ≤ 200 nm.

3. The emulsion as claimed in claim 1 or claim 2, in which at least 50% of the population by volume of the oil droplets has a size ≤ 110 nm.

4. The emulsion as claimed in any one of claims 1 to 3, in which the phase inversion occurs at a temperature of between 45°C and 80°C, preferably of between 50°C and 65°C.

5. The emulsion as claimed in any one of claims 1 to 4, also comprising at least one alditol.

6. The emulsion as claimed in claim 5, in which the alditol is sorbitol, mannitol, glycerol, xylitol or erythritol.

7. The emulsion as claimed in any one of claims 1 to 6, in which the aqueous phase is a buffered saline solution.

8. The emulsion as claimed in any one of claims 1 to 7, in which the TLR4 agonist is a chemical compound of formula I:

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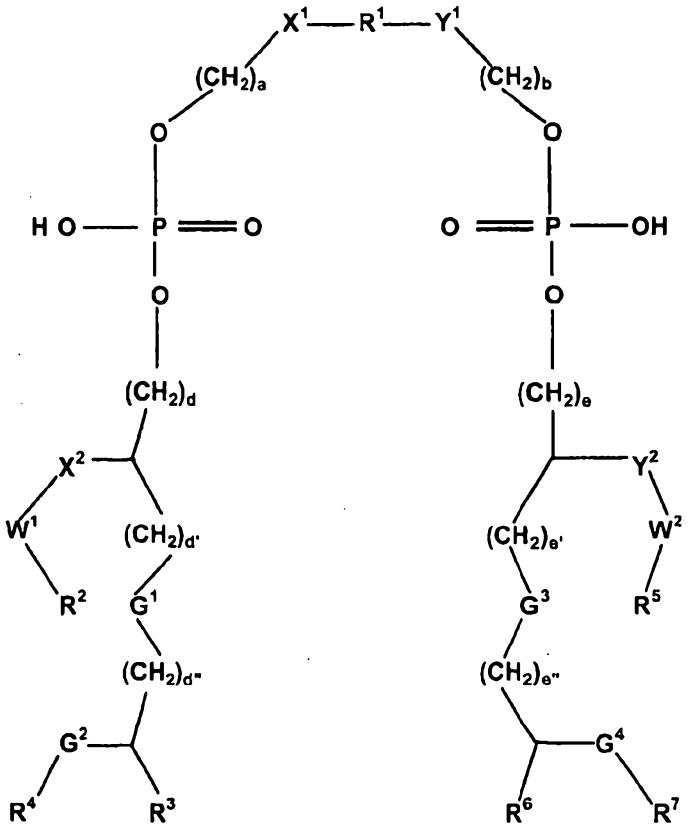
2007204295 22 Oct 2012

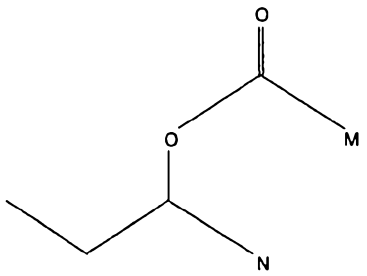


or a pharmaceutically acceptable salt of this compound.

9. The emulsion as claimed in claim 8, in which:
R1 is C(O) or C(O)-$(CH2)_n$-C(O), n being 1, 2, 3 or 4,
a, b, d, d', d'', e, e' and e'' are independently 1 or 2,
Xl, X2, Y1 and Y2 are NH,
W1 and W2 are C(O),

R2 and R5 are independently selected from the group consisting of a C_{10}-C_{15} straight chain alkyl optionally substituted with an oxo, an NH-(C_{10}-C_{15} straight chain alkyl), and

2007204295 22 Oct 2012



in which M and N are independently a C_2-C_{20} straight chain alkyl or alkenyl,
R3 and R6 are C_5-C_{10} straight chain alkyls,
R4 and R7 are selected from the group consisting of a hydrogen, C(O)-(C_8-C_{12} straight chain alkyl) and C(O)-(C_8-C_{12} straight chain alkenyl),
G1 and G3 are an oxygen or -NH(CO)-,
G2 and G4 are an oxygen.

10. The emulsion as claimed in either claim 8 or claim 9, in which the chemical compound is chosen from the group consisting of ER803022, ER803058, ER803732, ER803789, ER804053, ER804057, ER804058, ER804059, ER804442, ER804764, ER111232, ER112022, ER112048, ER112065, ER112066, ER113651, ER118989, ER119327 and ER119328.

11. The emulsion as claimed in any one of claims 1 to 10, in which the ratio of the amount of TLR4 agonist to the total amount of nonionic hydrophilic and hydrophobic surfactant is between $0.01x10^{-2}$ and $5x10^{-2}$, preferably between $0.05x10^{-2}$ and $2x10^{-2}$ (weight/weight).

12. The emulsion as claimed in any one of claims 1 to 11, in which the polyoxyethylene alkyl ether is chosen from the group consisting of polyoxyethylene (12) cetostearyl ether (ceteareth-12), polyoxyethylene (20) cetostearyl ether (ceteareth-20), polyoxyethylene (21) stearyl ether (steareth-21), polyoxyethylene (20) cetyl ether (ceteth-20), polyoxyethylene (10) cetyl ether (ceteth-10), polyoxyethylene (10) stearyl ether (steareth-10), polyoxyethylene (20)

2007204295 22 Oct 2012

stearyl ether (steareth-20), polyoxyethylene (10) oleyl ether (oleth-10) and polyoxyethylene (20) oleyl ether (oleth-20).

13. The emulsion as claimed in any one of claims 1 to 12, in which the hydrophobic surfactant is a sorbitan ester or a mannide ester.

14. The emulsion as claimed in claim 13, in which the hydrophobic surfactant is mannide monooleate.

15. The emulsion as claimed in claim 13, in which the hydrophobic surfactant is sorbitan monooleate.

16. The emulsion as claimed in any one of claims 1 to 15, in which the amounts of hydrophilic and hydrophobic surfactants are such that the overall HLB of the surfactants is between 8.5 and 10.

17. The emulsion as claimed in any one of claims 1 to 16, in which the amount of squalene represents between 5% and 45% of the total weight of the emulsion.

18. The emulsion as claimed in any one of claims 1 to 17, in which the ratio of the amount of squalene to the amount of surfactants is between 2.0 and 4.0, preferably between 2.5 and 3.5.

19. The emulsion as claimed in any one of claims 1 to 18, in which the TLR4 agonist is the chemical compound ER804057, the nonionic hydrophilic surfactant is polyoxyethylene (12) cetostearyl ether (ceteareth-12), the nonionic hydrophobic surfactant is sorbitan monooleate and the aqueous solvent is a phosphate buffer or a citrate buffer.

2007204295 22 Oct 2012

20. The emulsion as claimed in claim 19, in which:

a. the amount of squalene represents between 5% and 45% of the total weight of the emulsion (weight/weight),

b. the ratio of the amount of squalene to the total amount of polyoxyethylene (12) cetostearyl ether (ceteareth-12) and of sorbitan monooleate is between 2.0 and 4.0,

c. the amounts of ceteareth-12 and of sorbitan monooleate are such that the HLB is between 8.5 and 10, and

d. the ratio of the amount of ER 804057 to the total amount of polyoxyethylene (12) cetostearyl ether (ceteareth-12) and of sorbitan monooleate is between 0.01×10^{-2} and 2×10^{-2}.

21. The emulsion as claimed in claim 20, also comprising mannitol, the amount of which represents between 0.1 and 10% of the total weight of the emulsion.

22. The emulsion as claimed in any one of claims 1 to 21, also comprising a lyophilization substrate.

23. The emulsion as claimed in claim 22, in which the lyophilization substrate is an aqueous solution of sucrose, of mannitol and of dodecyl maltoside.

24. The use of an emulsion as claimed in any one of claims 1 to 23, for preparing a vaccine composition.

25. The use of an emulsion as claimed in any one of claims 1 to 23, for preparing a vaccine composition comprising, as vaccine antigen, at least one flu virus hemagglutinin.

26. The use of an emulsion as claimed in any one of claims 1 to 23, for preparing a vaccine composition comprising, as vaccine antigen, a cytomegalovirus (CMV) envelope antigen.

2007204295 22 Oct 2012

27. The use of an emulsion as claimed in claim 26, in which the CMV envelope antigen is the gB protein of CMV or a derivative thereof which comprises at least one neutralizing epitope.

28. The use of an emulsion as claimed in claim 27, in which the CMV envelope antigen is the gB protein from which the transmembrane domain has been deleted and in which the cleavage site is ineffectual.

29. A method for preparing an O/W emulsion as claimed in any one of claims 1 to 23, comprising a step in which a W/O inverse emulsion is obtained by raising the temperature and a step in which this W/O inverse emulsion is converted to an O/W emulsion by lowering the temperature.

30. The method as claimed in claim 29, in which the W/O emulsion is obtained by carrying out a first step in which an aqueous phase comprising an aqueous solvent, a polyoxyethylene alkyl ether and a TLR4 agonist is mixed with an oily phase comprising squalene and a nonionic hydrophobic surfactant so as to obtain an O/W emulsion, and a second step in which the O/W emulsion is heated to a temperature which is at least the phase inversion temperature of the emulsion.

31. The method as claimed in claim 29, in which the W/O emulsion is obtained by separately heating an aqueous phase comprising an aqueous solvent, a polyoxyethylene alkyl ether and a TLR4 agonist and an oily phase comprising squalene and a nonionic hydrophobic surfactant to a temperature which is at least the phase inversion temperature of the emulsion, and then by mixing the aqueous phase with the oily phase while at the same time keeping the temperature of the mixture at a temperature which is at least the phase inversion temperature.

32. The method as claimed in either claim 30 or claim 31, in which the TLR4 agonist is in the oily phase instead of being in the aqueous phase.

2007204295 22 Oct 2012

33. The method as claimed in any one of claims 30 to 32, in which the aqueous phase also comprises an alditol.

34. The method as claimed in any one of claims 30 to 33, in which the phase inversion temperature is between 45°C and 80°C, and preferably between 50°C and 65°C.

35. A method for preparing a vaccine composition, in which at least one vaccine antigen is mixed with an O/W emulsion containing a TLR4 agonist, the chemical structure of which does not comprise a sugar ring, characterized in that the O/W emulsion containing the TLR4 agonist is prepared according to a phase inversion method comprising a step in which an emulsion is obtained in the form of a W/O inverse emulsion by increasing the temperature and a step in which the W/O emulsion is converted to an O/W emulsion by lowering the temperature.

36. The method as claimed in claim 35, in which the O/W emulsion comprises:

a) a TLR4 agonist, as defined in claim 1,

b) squalene,

c) an aqueous solvent,

d) a nonionic hydrophilic surfactant which is a polyoxyethylene alkyl ether, and

e) a nonionic hydrophobic surfactant.

37. The method as claimed in any one of claims 29 to 36, also comprising a lyophilization step.

38. The emulsion as claimed in claim 1, substantially as hereinbefore described.