IMMUNOLOGICAL ADJUVANT EMULSION

The present invention relates to the field of vaccines; more particularly, the invention relates to the field of vaccines comprising an adjuvant emulsion.

Many vaccines which contain one or more adjuvants exist in the prior art. US patent 6 299 884 discloses in particular an adjuvant formulation comprising an oil-in-water emulsion, in which the size of the oil droplets is between 100 and 1000 nm. This emulsion is obtained by means of a high pressure homogenizer (microfluidizer), in the course of a manufacturing process using high mechanical energies in order to obtain shear forces that are sufficiently great to reduce the size of the oil drops. According to this teaching, while the minimum value of the size range of the drops obtained is 100 nm, the mean value is much higher and is, at best, in the region of 170 nm, more generally in the region of 500 nm.

It is desirable to have available a formulation that is an alternative to that proposed in that patent, that can be obtained by means of a simpler process (not requiring any specific shear technology) that is a low-energy process while at the same time being reproducible and completely reliable; in addition, the adjuvant formulation must make it possible to effectively adjuvant vaccines, by making it possible in particular to increase the immune response obtained or to decrease the dose of antigen present, while at the same time exhibiting no sign of toxicity that would be detrimental to its completely safe administration.

To achieve this aim, a subject of the present invention is an oil-in-water adjuvant emulsion characterized in that it comprises at least:

- squalene,
- an aqueous solvent,
- a hydrophilic nonionic surfactant which is a polyoxyethylene alkyl ether,
- a hydrophobic nonionic surfactant,

in that it is thermoreversible and in that 90% of the population by volume of the oil drop has a size less than 200 nm.

According to the invention, such an emulsion may be obtained by means of a temperature-variation phase-inversion process, which provides a very large advantage from an industrial point of view. Such a process provides all the guarantees of safety and of profitability necessary for the pharmaceutical industry. In addition, by virtue of

this process, it is possible to obtain a monodisperse emulsion, the droplet size of which is very small, which renders the emulsion thus obtained particularly stable and readily filterable by means of sterilizing filters, the cutoff threshold of which is 200 nm.

According to a particular characteristic, 90% of the population by volume (or d90) of the oil drops has a size less than 160 nm, and even less than 150 nm.

According to a particular embodiment of the invention, the emulsion according to the invention also comprises an alditol; this makes it possible to obtain a phase inversion at a temperature below that which would be necessary for the same composition not containing any alditol, which makes it possible to reduce the production costs and also the risks of thermal denaturation of the constituents of the emulsion.

According to a particularly advantageous embodiment, the hydrophobic nonionic surfactant of the invention is a sorbitan ester or a mannide ester. Such surfactants have the advantage of being able to be used entirely safely in injectable solutions.

According to a particular embodiment of the invention, the emulsion also comprises an alkylpolyglycoside and a cryoprotective agent such as a sugar, in particular dodecylmaltoside and/or sucrose.

Thus, it is possible to obtain a lyophilizable emulsion which, after lyophilization and reconstitution, recovers its properties, in particular particle size properties, i.e. the lyophilized and then reconstituted emulsion is still monodisperse and consists of oil droplets, 90% of the population by volume of which has a size of less than 200 nm. This is particularly important for the field of vaccines which must sometimes, for reasons of stability (either of certain antigens, or of certain adjuvants), be conserved in lyophilized form.

A subject of the invention is also a process for preparing an immunogenic composition according to which at least one vaccine antigen is mixed with an oil-in-water emulsion, characterized in that the oil-in-water emulsion is obtained by means of a temperature-variation phase-inversion process.

According to one embodiment, the process according to the invention comprises at least one step of preparing the oil-in-water emulsion by cooling a water-in-oil inverse emulsion, which comprises at least:

- squalene,
- an aqueous solvent,

- a hydrophilic nonionic surfactant which is a polyoxyethylene alkyl ether,
- a hydrophobic nonionic surfactant.

By virtue of such a process, which is very advantageous from an industrial point of view, a stable immunogenic composition is obtained which is very effective even at a very low dose of antigens.

In addition, by virtue of the preparation process used, the droplets of oil of the emulsion are all calibrated over the same very small size; in fact, when the particle size properties (size and size distribution) are measured, it is noted that the emulsion is a monodisperse emulsion, with a Gaussian-type distribution curve, which is very narrow and centered around a low value, generally around 80-90 nm.

According to a specific embodiment of the process according to the invention, the water-in-oil inverse emulsion is obtained by mixing squalene, an aqueous solvent, a hydrophilic nonionic surfactant which is polyoxyethylene alkyl ether, and a hydrophobic nonionic surfactant so as to obtain, first of all, an oil-in-water coarse emulsion, and this emulsion is then heated to at least the phase-inversion temperature so as to obtain the inverse emulsion. Carrying out the process in such a way has the advantage of limiting the time during which the various constituents of the emulsion are subjected to a high temperature.

According to another embodiment, the process according to the invention comprises the following steps:

- firstly, the aqueous phase comprising the aqueous solvent and the polyoxyethylene alkyl ether and, secondly, the oily phase comprising the squalene and the hydrophobic surfactant are heated, separately, to a temperature at least equal to the phase-inversion temperature, and then
- the 2 phases are mixed so as to obtain a water-in-oil inverse emulsion.

According to a specific embodiment of the invention, each of the aqueous and oily phases is heated separately, before mixing, to a temperature below the phase-inversion temperature. The 2 phases are then mixed so as to obtain a water-in-oil emulsion; the whole is then heated to a temperature at least equal to the phase-inversion temperature so as to obtain the water-in-oil inverse emulsion.

According to a particular embodiment, the preparation process according to the invention also comprises a lyophilization step. Thus, the process according to the invention can be used for the preparation of immunogenic compositions comprising antigens which must be conserved in lyophilized form for reasons of stability.

Many other advantages of the present invention will become apparent in the course of the following description.

For the purposes of description of the invention, the parameters d50 and d90 mentioned in the present patent application are values relating to volume; the d50 value signifies the value of 50% of the drop population by volume.

For the purpose of the invention, the term "oil-in-water emulsion" is intended to mean a dispersion of an oily phase in an aqueous phase which may consist either of water or of a saline solution, that is optionally buffered. According to a particular embodiment of the invention, the aqueous phase of the emulsion consists of a buffer, such as Dulbecco's phosphate buffered solutions (D-PBS, without calcium or magnesium). The term "adjuvant emulsion" is intended to mean an immunoadjuvant emulsion, i.e. an emulsion capable of modifying the response of the immune system induced during the administration of an antigen, compared with the response obtained in the absence of the emulsion; this immune system response may be reflected by antibody production or by activation of certain cells, in particular antigen-presenting cells (for example, dendritic cells), T lymphocytes and B lymphocytes. This cellular activation can be demonstrated by the presence of activation markers at the surface of the cells or by the release of cytokines. The modification of the immune response induced by the adjuvant emulsion may be quantitative in nature, i.e. an increase in the induced response is obtained, or qualitative in nature, i.e. a response that is different in nature or has a different orientation is obtained, or else an additional response is obtained. The term "adjuvant emulsion" is also intended to mean an emulsion that makes it possible to reduce the amount of antigens administered for the same induced response.

For the purpose of the present invention, the term "immunogenic composition" is intended to mean a composition comprising at least one antigen and that can be administered to humans or to animals in order to induce an immune system response. This response may be a humoral response (antibody production) or a cellular response (proliferation and/or activation of immune cells). The immunogenic composition may be a composition for prophylactic purposes or for therapeutic purposes, or else both. The immunogenic composition obtained according to the invention can be administered via any of the routes normally used or recommended for vaccines: parenterally or mucosally, and can be in various forms, in particular a liquid or lyophilized form. 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.

For the purpose of the present invention, the term "antigen" is intended to mean any antigen that can be used in a vaccine, whether it involves a whole microorganism or a subunit antigen, regardless of its nature; the antigen may in fact be a peptide, a protein, a glycoprotein, a polysaccharide, a glycolipid, a lipopeptide, etc.

The adjuvant emulsion according to the invention is particularly suitable for viral antigens; particularly good results have in fact been obtained with antigens of the human cytomegalovirus, of the human immunodeficiency virus, and of the flu virus. As regards the flu virus antigens, it is possible to use antigens that come from a single viral strain, or from a mixture of various strains. It is possible to use antigens derived from viruses cultured conventionally on eggs, or on cells. By means of the invention, it has been noted that whether for a single strain or for a mixture of strains, it is possible to obtain a satisfactory response of the immune system while at the same time very substantially reducing the amount of antigens present in the vaccine dose. This may of particularly great value in the case of the preparation of a vaccine against a flu pandemic, where it must be possible to produce, in a very short period of time, very large amounts of vaccine doses.

According to the invention, the oil-in-water emulsion comprises squalene, which is an oil initially originating from shark liver; it is an oil whose empirical chemical formula is $C_{30}H_{50}$, comprising 6 double bonds; this oil is metabolizable and has qualities that allow it to be used in an injectable pharmaceutical product. Squalene of plant origin,

extracted from olive oil, also exists. Good results have in particular been obtained using the squalene provided by the company Fluka, which is of animal origin. The amounts of squalene used for the preparation of a concentrated emulsion are advantageously between 5 and 45%; this concentrated emulsion is subsequently diluted during the preparation of immunogenic compositions so as to prepare immunizing doses in which the amount of squalene is between 0.5 and 5%. This dilution can be carried out by simply mixing the adjuvant emulsion according to the invention and the suspension comprising the antigen.

According to the invention, the emulsion comprises a nonionic hydrophilic surfactant, the value of the hydrophilic/lipophilic balance or HLB of which is greater than or equal to 10 and which belongs to the chemical group of polyoxyethylene alkyl ethers (PAEs), also known as polyoxyethylenated fatty alcohol ethers, or n-alkyl polyoxyethylene glycol 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 $CH_3(CH_2)_x$-$(O$-CH_2-$CH_2)_n$-OH in which n denotes the number of ethylene oxide units and is usually between 10 and 60, and $x + 1$ is the carbon number, depending on the fatty alcohols used. In general, these products are mixtures of polymers with hydrocarbon-based chains of similar length. The emulsion according to the invention usually comprises a single hydrophilic PAE. A mixture of several PAEs is also suitable insofar as the overall HLB value is ≥ 10. The polyoxyethylenated fatty alcohol ethers suitable for the subject of the invention can be in a liquid or solid form 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, myristyl, cetyl, oleyl and/or stearyl alcohols 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® for the products sold by the company ICI America's Inc., Eumulgin® for the products sold by the company Cognis, or Simulsol® for the products sold by the company SEPPIC.

A particularly preferred emulsion according to the invention contains, as nonionic hydrophilic surfactant, a polyoxyethylene alkyl ether chosen from the group consisting of ceteareth-12 (sold under the name Eumulgin® B1), ceteareth-20 (Eumulgin® B2), steareth-21 (Eumulgin® S21), ceteth-20 (Simulsol® 58 or Brij® 58), ceteth-10 (Brij® 56), steareth-10 (Brij® 76), steareth-20 (Brij® 78), oleth-10 (Brij® 96 or Brij® 97) and oleth-20 (Brij® 98 or Brij® 99). The number assigned to each chemical name corresponds to the number of ethylene oxide units in the chemical formula.

Good results have been obtained with the product Brij® 56. A compound that is particularly suitable and preferred due to its semisynthetic origin is the polyoxyethylene (12) cetostearyl ether supplied by the company Cognis under the name Eumulgin™B1. This compound is a mixture of $CH_3(CH_2)_{15}$-$(O\text{-}CH_2\text{-}CH_2)_{12}$-OH and of $CH_3(CH_2)_{17}$-$(O\text{-}CH_2\text{-}CH_2)_{12}$-OH.

According to the invention, the adjuvant emulsion also comprises a hydrophobic nonionic surfactant; it must be a surfactant that can be used in the pharmaceutical industry; among surfactants that are suitable in this regard, mention may be made of sorbitan esters and mannide esters; the sorbitan esters are obtained by reaction of a fatty acid and of a mixture of partial esters of sorbitol and its mono- and dianhydrides; this may involve a mono-, a di- or a triester, or even a mixture; they are hydrophobic surfactants for which the overall hydrophilic-lipophilic balance (HLB) is less than 9, and preferably less than 6. They can be found in particular in the range of surfactants sold by the company ICI Americas Inc. under the name Span®, or by the company Cognis under the name Dehymuls™, or by the company ICI under the name Arlacel™; as examples of surfactants that are particularly suitable, mention may be made of the sorbitan monooleate sold under the name Dehymuls SMO™ or Span®80. Among the surfactants consisting of mannide esters, mention may be made of the mannide monooleate sold by the company Sigma, or by the company Seppic under the name Montanide 80™.

By virtue of the selection of these specific surfactants among all the surfactants proposed in the prior art for preparing emulsions, it has now been found that it is

possible, very advantageously, to produce an oil-in-water adjuvant emulsion using a phase-inversion process.

For this, the amounts of squalene and of each of the surfactants used are advantageously chosen so as to obtain a mixture, the phase diagram of which comprises a zero mean curvature phase (microemulsion or lamellar phase type) for which the interfacial tensions are extremely low.

In the case of the use of squalene, it has been noted that the emulsions obtained are stable and monodisperse, with the oil droplets being very small in size (d90 less than 200 nm), when the overall HLB value of the various surfactants used is between 8.5 and 10, and more particularly 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 can be used:

$HLB_m = (HLB_e \times M) + HLB_{pae}\,(1\text{-}M)$ 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 mass of the hydrophobic surfactant in the mixture consisting of the hydrophobic surfactant and the polyoxyethylene alkyl ether (PAE),

HLB_{pae} corresponds to the HLB of the PAE.

It has been noted that, by using a concentration of squalene of between 5 and 45%, an emulsion whose phase-inversion temperature is less than 95°C is very advantageously obtained.

For such an emulsion, it is possible to use a polyoxyethylene alkyl ether at a concentration of between 0.9 and 9% and a hydrophobic nonionic surfactant at a concentration of between 0.7 and 7%; the remainder of the emulsion consisting of an aqueous solvent.

According to a particular embodiment of the invention, the immunogenic composition also comprises an alditol such as, in particular, glycerol, erythritol, xylitol, sorbitol or

mannitol. Good results have in particular been obtained with the mannitol sold by the company Roquette Frères. The amounts of alditol used in the preparation process can range between 1 and 10%, and more particularly between 2 and 7.

According to a particular embodiment of the invention, the adjuvant emulsion also comprises a cryoprotective agent which makes it possible to lyophilize the emulsion obtained; among the cryoprotective agents, sugars are particularly preferred, and especially sucrose. In addition, the emulsion according to the invention can comprise an alkylpolyglycoside, which is a surfactant with a sugar head; it may in particular be sodium decyl-D-galactoside uronate, or, according to a preferred embodiment, dodecyl-β-maltoside available from the company Roche.

By virtue of the process for preparing the emulsion according to the invention by phase inversion obtained by varying the temperature, an oil-in-water emulsion, the size of the oil droplets of which is very homogeneous, is very readily and very reproducibly obtained: the d90 value (by volume) is less than 200 nm, preferably less than 150 nm, and even close to 100 nm while the d50 value is less than 100 nm, or even 90 nm. Most of the emulsions prepared according to the process of the invention have made it possible to attain d50 values of around 80 nm, with d90 values of around 100 nm (measurements carried out with a Coulter LS230). It is thus possible to perform a sterilizing filtration of the emulsion obtained, on the condition that the latter is sufficiently diluted.
Such emulsions in which the size of the drops is homogeneous and very small are stable over time. It has thus been possible to note that an emulsion prepared according to the invention and stored at 4°C, conserved, after 2 years, a monodisperse profile with a d50 value of 90 nm and a d90 value of 116 nm, which proves that the emulsion is very highly stable.

The size of the drops can be measured by various means, and in particular by LASER diffraction particle sizes, such as the Beckman Coulter devices of the LS range (in particular the LS230) or Malvern devices of the Mastersizer range (the Mastersizer 2000 in particular). 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 sample (here, 1.495 for squalene) and of its medium (here, 1.332 for water); it is also necessary to be capable of detecting the weak intensities emitted by the very fine particles, which requires an optimization of the analysis:

- 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 blue light of shorter wavelength, associated with wide-angle scattering and backscattering detectors, reinforces the performance levels of the analysis in the submicronic range.

According to the devices used, the measurements may vary slightly as a function of the components of the device and of the data processing software used. Thus, the same emulsion according to the invention was analyzed with the 2 devices and gave the following results:

- using the LS230, with the following parameters: IR particle = 1.495; IR medium = 1.332; absorption value = 0; d50 = 80-90 nm and d90 = 120-130 nm;
- using the Mastersizer 2000, with the following parameters: IR particle = 1.495; IR medium = 1.332; absorption value = 0; obscuring = 4-7%; "general purpose" optical model; d50 = 90-100 nm and d90 = 140-150 nm.

The process according to the invention may be carried out in the following way: a concentrated crude oil-in-water emulsion is prepared by incorporation of the aqueous phase (buffer solution, to which alditol is optionally added, comprising the polyoxyethylene alkyl ether) into the oily phase (squalene and hydrophobic nonionic surfactant); or, conversely, by incorporation of the oily phase into the aqueous phase. A noncalibrated oil-in-water emulsion is then obtained, which rapidly manifests its instability. This emulsion is stirred and heated until a phase inversion is obtained, i.e.

a water-in-oil emulsion is obtained. The phase inversion or transition can be monitored by conductimetry. In fact, during the rise in temperature, the conductivity increases until phase inversion occurs; at this time, a relatively abrupt drop in conductivity is observed. The temperature at which the change in curvature of the curve for following the conductivity occurs reflects the passage from one type of emulsion to another; it is the phase inversion temperature. In reality, this temperature is rather a temperature range than a very precise point value; in fact, it may be considered that this temperature is a temperature determined to within 1 or 2 degrees so that the entire emulsion undergoes the phase inversion phenomenon. Once this phase inversion temperature has been reached, and therefore once in the presence of a water-in-oil emulsion, the heating is stopped and the mixture is cooled. The cooling can be carried out passively, by simply allowing the emulsion to return spontaneously to ambient temperature, or more actively, by immersing the emulsion, for example, in an ice bath. When the temperature passes through the phase inversion temperature, the water-in-oil emulsion will invert again so as to once more give an oil-in-water emulsion, in which the size of the oil droplets is this time very homogeneous and small; the emulsion obtained is then very stable. It can be stored as it is, while awaiting dilution with a solution comprising the vaccine antigen.

This emulsion is thermoreversible, which means that, if it is again brought to a temperature above the phase inversion temperature, it will once again become a water-in-oil emulsion. It is noted that the curves for following the conductivity are superimposable for the same emulsion, irrespective of the number of thermoinversions undergone, and that the emulsions obtained always have the same particle size profile.

Advantageously according to the invention, the formulation of the emulsion is chosen so as to have a phase inversion temperature that is less than 95°C, and more particularly between 45 and 80°C, and more particularly still between 50 and 65°C. This temperature range is advantageous since there is no risk of the emulsion changing state if it is stored at a relatively high temperature (≈ 37°C). Furthermore, as in the process for preparing the thermoreversible emulsion, the heating of the components is not too severe, this contributes to maintaining the structural integrity of the components. When the phase-inversion temperature of the emulsion is high, in particular when it is greater than or in the region of 80°C, it may be useful to reduce it

by adding to the composition of the emulsion an alditol, which is normally chosen from sorbitol, mannitol, glycerol, xylitol or erythritol. When the alditol is used 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 is reduced by approximately 10°C. It is also possible to reduce the phase-inversion temperature of the emulsion by replacing the aqueous phase consisting only of water with a buffered saline aqueous phase. A Tris buffer, or a phosphate buffer such as PBS, or the Dulbecco PBS buffer without Ca^{2+} and without Mg^{2+}, is normally used.

Alternatives to the process that has just been described exist. Specifically, it is possible, as has just been described, to mix the 2 aqueous and oily phases in order to obtain the crude emulsion which will then be heated and then cooled.
Alternatively, the 2 phases that have been prepared can be heated separately to a temperature slightly above the phase inversion temperature, before being mixed so as to give a water-in-oil inverse emulsion, which will be cooled until the oil-in-water submicronic emulsion is obtained.
It is also possible to slightly heat each of the phases before carrying out the mixing, which will result in an oil-in-water emulsion, and then to heat this emulsion to phase-inversion before carrying out the cooling.

All these operations can be carried out in separate containers for a batch preparation but it is also possible to use an on-line process.

The process for preparing the emulsion on-line may notably consist of a mixing, under hot conditions, of the two aqueous and oily phases prepared separately beforehand, 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 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 made up of cross blades inclined relative to the axis of the tube into which they are introduced was successfully used. The energy required for the mixing is provided by the pumps that transport the fluids and the mixing is carried out without any mobile part, through

the mixing elements by virtue of the separating, displacing and successive combining of the constituents of the mixture.

The on-line production process is carried out in the following way: the aqueous phase (buffered solution comprising the polyoxyethylene alkyl ether) and the oily phase (squalene and hydrophobic nonionic surfactant) are prepared separately 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 water-in-oil inverse emulsion is obtained during the passage of the two phases in the static mixer. The inverse emulsion is subsequently cooled by passing it on-line through a refrigerated heat exchanger connected at the outlet of the static mixer. The water-in-oil emulsion will then invert through the refrigerated heat exchanger to give an oil-in-water emulsion, which will be collected in a flask or reactor, and the characteristics of which are identical to those of the emulsion obtained by a batch process.

The adjuvant emulsion according to the invention is then used for the preparation of an immunogenic composition. A simple embodiment consists in mixing a solution comprising at least one vaccine antigen with an emulsion obtained according to one of the embodiments which have just been described. The immunogenic composition obtained is in the form of an oil-in-water emulsion or in the form of a thermoreversible oil-in-water emulsion when the amount of squalene represents at least 5% by mass of the total mass of the immunogenic composition. Alternatively, it is possible to mix the antigen with the aqueous phase or with the oily phase before preparing the emulsion. Carrying out the process in such a manner implies, of course, that said antigens are antigens which are compatible with the thermoinversion process.

The solutions of the antigen may also contain mineral salts or one or more buffers, and also any other compound normally used in vaccines, such as stabilizers, preserving agents or, optionally, also other adjuvants.

For the preparation of a lyophilizable emulsion, a concentrated liquid emulsion is first of all prepared, as has just been described, but preferably choosing water rather than a

buffered solution as aqueous phase, and then this emulsion is diluted with a solution comprising an alditol, a sugar and an alkylpolyglycoside, for example with a solution comprising mannitol, sucrose and dodecylmaltoside.

The emulsion obtained 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,
- approximately 2 hours of freezing at a set temperature of -45°C,
- 14 to 19 hours of primary desiccation at a set temperature of 0°C,
- 3 hour 30 min of secondary desiccation at a set temperature of + 25°C.

The emulsion obtained can then be conserved until it is used for the preparation of an immunogenic composition, i.e. until it is combined with a composition comprising vaccine antigens. This step for preparing the immunogenic composition can be carried out by taking up the lyophilized emulsion with an aqueous solution comprising the antigens. The immunogenic composition thus obtained can subsequently be conversed in the liquid state, or can be subjected to a further lyophilization cycle in order to be conserved in the form of a lyophilisate, 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 also the alditol, the sugar and the alkylpolyglycoside, and to subsequently subject the composition obtained to the lyophilization. Such a manner of carrying out the process implies, of course, that the antigens are antigens that are compatible with a lyophilization process.

The following examples illustrate various embodiments of the invention.

Example 1: Preparation of an adjuvant emulsion according to the invention

3.71 g of Eumulgin™ B1 and 33.9 g of a 10% solution of mannitol in PBS buffer were mixed in a beaker, and the mixture was homogenized with stirring at approximately 30°C.

In another container, 2.89 g of Dehymuls™ SMO and 19.5 g of squalene were stirred magnetically.

When homogeneous phases were obtained in each of the containers, the aqueous phase was incorporated into the oily phase, which was maintained at 30°C with stirring.

When the incorporation was complete, the crude emulsion obtained was heated until the temperature reached 58-60°C, while at the same time maintaining the stirring. The heating was then stopped but the stirring was maintained until the temperature reached ambient temperature.

An oil-in-water emulsion was then obtained, the size of the oil droplets of which was centered around 80 nm (measurement carried out using an LS230), and the composition by mass of which was as follows:

- 32.5% of squalene,
- 6.18% of polyoxyethylene (12) cetostearyl ether,
- 4.82% of sorbitan monooleate,
- 6% of mannitol.

Example 2: Vaccine composition against AIDS.

Vaccine compositions comprising a detoxified TAT III B protein as antigen were prepared. The TAT protein was detoxified by means of an alkylation reaction in an alkaline medium using iodoacetamide under the following conditions: number of micromoles of iodoacetamide = 200 X number of micromoles of TAT + number of micromoles of DTT. This detoxified protein and the process for preparing it are described in detail in application WO99/33346, where it is identified under the term carboxymethylated TAT. This recombinant TAT antigen is conserved in solution, in the presence of 50 mM Tris buffer, pH 7.5, at –70°C.

The vaccine compositions to be administered were prepared from concentrated solutions, in order to obtain immunization doses of 200 µl having the following quantitative compositions:

- for the composition having only the antigen: 20 µg of TAT in 50 mM Tris buffer, 100 mM NaCl, at pH 7.5;
- for the composition according to the invention:
  - 20 µg of TAT,

- 5 mg of squalene
- 0.75 mg of Dehymuls SMO,
- 0.94 mg of Eumulgin B1,
- 0.91 mg of mannitol.

Two groups of six 8-week-old female BALB/c mice were provided, and were injected subcutaneously with one of the compositions prepared, in a proportion of one dose of 200 μl per mouse; the injections were given on D0 and on D21.

Blood samples were taken from the retroorbital sinus on D14 in order to assess the primary response and on D34 for the secondary response. The specific IgG1 and IgG2a titers were determined by means of standardized ELISA assays.

The mice were sacrificed on D37; their spleen was removed and the splenocytes were isolated.

The results obtained regarding the humoral responses are summarized in the table below, in which the IgG titers are expressed in arbitrary ELISA units (log10).

For each group of mice, the value indicated in the table is the mean geometric titer of the values obtained for each of the mice.

Vaccine composition	IgG1 at D14	IgG2a at D14	IgG1 at D34	IgG2a at D34	IgG1/IgG2a ratio at D34
Tat	2.6	1.2	4.4	3.0	25
Tat+ PIT	3.5	2.5	5.7	5.0	5

The results obtained show that the emulsion according to the invention makes it possible to increase, overall, the humoral response and also tends to promote the type 1 T-helper response since the IgG2a response is increased more than the IgG1 response.

As regards the cellular response, it was possible to demonstrate, by ELISPOT assay after restimulation of the splenocytes removed with the recombinant TAT protein, a clear increase in the number of cells producing γ interferon when the splenocytes came from mice immunized with a preparation according to the invention (486 spots per 10^6 cells versus 39 per 10^6 for the preparation containing the antigen alone). Similarly, the assaying of the cytokines in the culture supernatants showed the greater secretion of both γ interferon (5028 pg/ml versus 1940 pg/ml) and Interleukin 5 (5365 pg/ml versus 2394 pg/ml).

Example 3: Preparation of a vaccine composition against human cytomegalovirus infections.

Vaccine compositions comprising, as vaccine antigen, a recombinant protein derived from an envelope glycoprotein of the Cytomegalovirus (CMV) Towne strain, called gB, the nucleotide and protein sequences of which are described in patent USP 5,834,307, were prepared. This recombinant protein is produced by a recombinant CHO line transfected with a plasmid called pPRgB27clv4 which contains a modified gB gene. Specifically, in order to facilitate the production of this recombinant protein by the CHO line, the gB gene was modified beforehand by deleting the part of the gene that encodes the transmembrane region of the gB protein corresponding to the amino acid sequence between Valine 677 and Arginine 752 and by introducing 3 point mutations such that the cleavage site that exists in the native gB was eliminated. In fact, the recombinant protein produced by the recombinant CHO line corresponds

to a truncated gB protein devoid of cleavage site and of transmembrane region, called gBdTM.

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 purification of the truncated gB protein is carried out on an immunoaffinity chromatographic column using the monoclonal antibody 15D8 described by Rasmussen L. et al. (J. Virol. (1985) 55: 274-280).

From a stock composition at 0.975 mg/ml of gB antigen thus obtained and maintained in phosphate buffer, the emulsion according to the invention concentrated as described in example 1, and an emulsion of the prior art obtained by microfluidization, 50 μl doses of immunizing compositions were prepared, having the following compositions:

- 2 μg of gB in citrate buffer at pH 6 (group called gB alone),
- 2 μg of gB; 1.075 mg of squalene; 0.133 mg of Montane™ VG 85 and 0.125 mg of Tween™80 in citrate buffer at pH 6 (group called «with emulsion of the prior art»),
- 2 μg of gB; 1.25 mg of squalene; 0.185 mg of Dehymuls SMO; 0.235 mg of Eumulgin™ B1 and 0.230 mg of mannitol in PBS buffer at pH 7.4 (group called «with emulsion of the invention»).

Three groups of ten 8-week-old female Outbred OF1 mice were provided and were immunized twice, on D0 and on D21, subcutaneously, with one of the compositions indicated above (each group of mice is given the same composition both times). Blood samples were taken from the retroorbital sinus on D20 and D34 and were used to determine the concentrations of IgG1- and IgG2a-type antibodies specific for the gB antigen.

The assays were carried out by means of ELISA assays; the results obtained are given in the table below, and are expressed as $\log_{10}$ of the ELISA titers. The values indicated are the mean values obtained for each group of mice.

Nature of the groups	D20		D34		IgG1/IgG2a ratio at D34
	IgG1	IgG2a	IgG1	IgG2a	
gB alone	2.474	2.094	3.801	2.941	137
gB + emulsion of the prior art	4.063	2.980	5.493	4.185	143
gB + emulsion according to the invention	4.615	3.914	5.615	4.854	14

These results show the effectiveness of the emulsion according to the invention, which allowed a greater induction of antibodies, both those of IgG1 type and of IgG2a type, with, in addition, compared with the emulsion according to the prior art, the obtaining of the desired result in the context of a vaccine against human cytomegalovirus, which is that of orienting the immune response toward a TH1 response (an indicator of which is the IgG2a titer), while at the same time maintaining the TH2-type response (an indicator of which is the IgG1 titer) at a sufficient level.

Example 4: Vaccine composition against the flu.

Flu virus antigens were available, obtained according to the process described in the examples of application WO 96/05294, with the exception of the fact that the viral strain used was the A/New Caledonia H1N1 strain.

Using this preparation of antigens, the concentrated emulsion according to the invention obtained in example 1, and a suspension of aluminum provided by REHEIS under the name AlOOH-Rehydra, 50 µl immunization doses were prepared, which doses had the composition indicated hereinafter, in which the amounts of flu antigens are expressed by weight of hemagglutinin HA:

- either 1 µg of HA in PBS buffer,
- or 5 µg of HA in PBS buffer,
- or 1 µg of HA and 60 µg of aluminum hydroxide,
- or 1 µg of HA; 1.25 mg of squalene; 0.185 mg of Dehymuls™ SMO; 0.235 mg of Eumulgin™ B; 0.21 mg of mannitol; the entire mixture in PBS buffer.

Eight groups of five 8-week-old female BALB/c mice were provided and were administered, on D0, with the compositions prepared according to the following distribution:

- one group received the composition having 1 μg of HA, subcutaneously,
- one group received the same composition having 1 μg of HA, intradermally,
- one group received the composition having 5 μg of HA, subcutaneously,
- one group received the same composition having 5 μg of HA, intradermally,
- one group received the composition having 1 μg of HA and 60 μg of aluminum, subcutaneously,
- one group received the same composition having 1 μg of HA and 60 μg of aluminum, intradermally,
- one group received the composition having 1 μg of HA and the emulsion according to the invention, subcutaneously,
- one group received the composition having 1 μg of HA and the emulsion according to the invention, intradermally.

Blood samples were taken from each of the mice on D14, D28, D41, D56 and D105. The sera from the immunized mice were assayed, firstly, by the ELISA technique in order to evaluate their content of total antibodies induced against the flu strain A/H1N1 of the trivalent vaccine (the antibody titers are expressed as the log10 value of arbitrary ELISA units, with a detection threshold of 1.3 log10) and, secondly, by the HAI (hemaglutination inhibition) technique in order to determine their content of functional antibodies against the flu strain A/H1N1. The antibody titers are expressed as the inverse of the dilution in arithmetic value, with a detection threshold at 5.

The results obtained are reiterated in the tables hereinafter, in which the values indicated represent the mean of the titers of the mice of each group.

ELISA titers:

Group of mice	Immunization route	D14	D28	D41	D56	D105
HA 1 μg	subcutaneous	2.838	3.288	3.556	3.581	3.535
HA 5 μg	subcutaneous	3.203	3.642	3.836	3.732	3.844
HA 1 μg + AlOOH	subcutaneous	2.860	3.363	3.843	3.951	3.982
HA 1 μg + emulsion	subcutaneous	4.043	4.511	4.753	4.723	4.681
HA 1 μg	intradermal	2.174	2.814	3.143	3.075	2.735
HA 5 μg	intradermal	2.839	3.297	3.496	3.549	3.479
HA 1 μg + AlOOH	intradermal	2.654	3.004	3.137	3.020	2.724
HA 1 μg + emulsion	intradermal	4.134	4.692	4.895	4.911	4.823

HAI titers:

Group of mice	Immunization route	D14	D28	D41	D56	D105
HA 1 μg	subcutaneous	5	6	5	40	23
HA 5 μg	subcutaneous		5	9	20	
HA 1 μg + AlOOH	subcutaneous	5	15	23	121	160
HA 1 μg + emulsion	subcutaneous	6	160	368	422	485
HA 1 μg	intradermal	5	5	5	23	8
HA 5 μg	intradermal		5	8	10	
HA 1 μg + AlOOH	intradermal	5	5	5	26	7
HA 1 μg + emulsion	intradermal	7	557	1640	1557	1844

These results show the advantage of the present invention; specifically, aluminum hydroxide, which is a well known adjuvant widely used in the prior art, did not make it possible to increase the immunogenicity of the flu antigens with the same rapidity and the same strength as the formulation according to the invention; it is also noted that the composition according to the invention was particularly effective, whether given subcutaneously or intradermally.

Example 5: Vaccine composition against the flu.

The intention was to evaluate, in mice, the advantage of the present invention for decreasing the amount of vaccine antigen when the vaccine involved is a vaccine against the flu which contains, as antigens, 3 flu virus strains and which would be administered intradermally.

To this end, the concentrated emulsion of example 1 was diluted with PBS buffer in order to obtain a 5% squalene emulsion, which was further diluted by half with a composition comprising the antigens.

A composition comprising flu virus originating from 3 different viral strains obtained in the manner described in patent application WO 96/05294 was in fact available, the 3 strains being in this case the A/New Caledonia (H1N1) strain, the A/Wyoming (H3N2) strain and the B/Jiangsu strain. Such a trivalent vaccine composition is conventional for a flu vaccine and corresponds to the vaccine sold in the northern hemisphere during the 2004 flu campaign. The amounts of antigens of each of the viral strains are assessed through their amount of hemagglutinins HA.

The immunization doses prepared, having a volume of 50 μl, had the compositions indicated below:

- 0.33 μg of HA of each of the viral strains in PBS buffer at pH 7.4;
- 1.31 μg of HA of each of the viral strains in PBS buffer at pH 7.4;
- 5.25 μg of HA of each of the viral strains in PBS buffer at pH 7.4;
- 10.5 μg of HA of each of the viral strains in PBS buffer at pH 7.4;
- 21 μg of HA of each of the viral strains in PBS buffer at pH 7.4;
- 0.33 μg of HA of each of the viral strains; 1.25 mg of squalene; 0.185 mg of Dehymuls™ SMO; 0.235 mg of Eumulgin™ B1 and 0.230 mg of mannitol in PBS buffer at pH 7.4;

- 1.31 µg of HA of each of the viral strains; 1.25 mg of squalene; 0.185 mg of Dehymuls™ SMO; 0.235 mg of Eumulgin™ B1 and 0.230 mg of mannitol in PBS buffer at pH 7.4;
- 5.25 µg of HA of each of the viral strains; 1.25 mg of squalene; 0.185 mg of Dehymuls™ SMO; 0.235 mg of Eumulgin™ B1 and 0.230 mg of mannitol in PBS buffer at pH 7.4.

Eight groups of ten 6- to 8-week-old female BALB/c mice were provided and were administered intradermally (internal face of the ear) with one of the compositions prepared, at a rate of one composition per group.

Three weeks after immunization, blood samples were taken and, for each of the groups, the IgGs induced against each of the viral strains were assayed by ELISA; a hemagglutinin inhibition assay against each viral strain was also carried out for each of the groups.

The results obtained are represented in the table below in the form of means for each of the groups. The ELISA results are expressed as $\log_{10}$ of arbitrary units, and the HAI results are the mean arithmetic titers of the inverses of the dilutions.

Nature	H1N1		H3N2		B	
Composition	ELISA	HAI	ELISA	HAI	ELISA	HAI
0.33 µg HA	3.51	35	4.38	243	4.28	25
1.31 µg HA	3.96	65	4.74	640	4.35	32
5.25 µg HA	4.16	92	5.05	970	4.62	53
10.5 µg HA	4.38	197	5.18	1810	4.71	130
21 µg HA	4.63	260	5.37	2389	4.98	184
0.33 µg HA + emulsion	4.27	226	5.20	2389	4.91	149
1.31 µg HA + emulsion	4.46	279	5.37	3880	4.95	171
5.25 µg HA + emulsion	4.68	394	5.58	5487	5.12	22

These results demonstrate the particular advantage of the invention for reducing the amount of antigens; specifically, by virtue of the emulsion according to the invention, it was possible, for the same immune response induced, to very substantially decrease the amount of antigens present in the immunization dose.

Example 6: Trivalent vaccine composition against the flu in a non-naive population

The intention was to test the effectiveness of the emulsion of the invention in the case of a flu vaccine that would be administered to individuals whose body has already been in contact with flu virus antigens, as is frequently the case, either because the individuals have already been in contact with the flu virus, or because they have already been previously immunized with a flu vaccine.

According to the information published by C.W. Potter in *Vaccine, 2003, 21:940-5,* it is possible to use, as animal model for carrying out this test, BALB/c mice pre-immunized intramuscularly with a trivalent vaccine.

50 µl immunization doses comprising either PBS buffer only, or trivalent vaccine from the 2004 campaign, i.e. a vaccine comprising the A/New Caledonia (H1N1) strain, the A/Wyoming (H3N2) strain and the B/Jiangsu strain, in a proportion of 5 µg of HA of each of the strains, in PBS buffer at pH 7.4, were therefore prepared.

Six groups of seven BALB/c mice were provided; 3 groups were immunized with the doses comprising only buffer, and 3 others with the trivalent vaccine, intramuscularly.

30 µl immunization doses were also prepared from the concentrated emulsion of example 1 and a vaccine composition comprising the 3 viral strains of the 2004 campaign mentioned above, these immunization doses having the following compositions:

- PBS buffer alone,
- 0.3 µg of HA of each of the viral strains in PBS buffer,
- 0.3 µg of 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 in PBS buffer at pH 7.4.

Each of the compositions thus prepared was used to immunize, on D34 intradermally (in the internal face of the ear), both a group of mice having previously received only PBS buffer, and a group of mice having received a dose of trivalent vaccine.

On D56, a blood sample was taken from each of the mice and the antibodies produced against the H1N1 strain (A/New Caledonia) were titered by means of a hemagglutinin inhibition assay.

The results obtained have been summarized in the table below and represent the mean values obtained for each group of mice having followed the same immunization protocol.

Mouse group identification	Nature of the dose for the im priming	Nature of the dose for the id boost	HAI titer with respect to the H1N1 strain
A	PBS	PBS	5
B	PBS	Trivalent vaccine containing 0.3 µg HA/strain	59
C	PBS	Trivalent vaccine containing 0.3 µg HA/strain + emulsion	320
D	Trivalent vaccine containing 5 µg HA/strain	PBS	145
E	Trivalent vaccine containing 5 µg HA/strain	Trivalent vaccine containing 0.3 µg HA/strain	476
F	Trivalent vaccine containing 5 µg HA/strain	Trivalent vaccine containing 0.3 µg HA/strain + emulsion	861

These results show how advantageous the invention is, even in individuals who are non-naïve with respect to the antigen administered. In fact, contrary to the

observations by C.W. Potter when using this model, who, himself, has only been able to show a weak adjuvant effect of Iscoms when they were used to immunize mice pre-infected or pre-immunized with flu antigens, here it is seen that the emulsion according to the invention made it possible to significantly increase the response induced, whether this was with naïve mice or with mice having already been immunized with flu vaccine.

Example 7: Trivalent vaccine composition against the flu comprising low doses of antigens

30 μl immunization doses were prepared from the concentrated emulsion of example 1 and a vaccine composition comprising the 3 viral strains of the 2004 campaign (the A/New Caledonia (H1N1) strain, the A/Wyoming (H3N2) strain and the B/Jiangsu strain), these immunization doses having the following compositions:

- 0.1 μg of HA of each of the viral strains in PBS buffer,
- 0.4 μg of HA of each of the viral strains in PBS buffer,
- 1.6 μg of HA of each of the viral strains in PBS buffer,
- 6.3 μg of HA of each of the viral strains in PBS buffer,
- 0.1 μg of HA; 0.75 mg of squalene; 0.11 mg of Dehymuls™ SMO; 0.143 mg of Eumulgin™ B1 and 0.138 mg of mannitol in PBS buffer at pH 7.4,
- 0.4 μg of HA; 0.75 mg of squalene; 0.11 mg of Dehymuls™ SMO; 0.143 mg of Eumulgin™ B1 and 0.138 mg of mannitol in PBS buffer at pH 7.4.

Six groups of eight 8-week-old female BALB/c mice were provided and were administered, on D0, intradermally (internal face of the ear) with a dose of 30 μl of one of the compositions indicated below (1 composition per group).
In each group, a 2nd dose having the same nature as the 1st dose administered was again administered, intradermally, to half the mice on D29.
Blood samples were taken on D22 and on D43 in order to determine the amounts of antibodies induced.
The antibody titers were assayed by ELISA for the antibodies induced at D22 and at D43, with respect to all the strains administered: H1N1, H3N2 and B, and by HAI with respect to the H1N1 strain only, both at D22 and at D43. The results obtained have been summarized in the table below, in which the titers expressed are the means

obtained for each group of mice. As regards the results at D43, the means were determined separately within the same group, for the mice having received 2 doses of vaccine and those having received only one.

Vaccine composition	H1N1 ELISA titer at D22	H3N2 ELISA titer at D22	B ELISA titer at D22	H1N1 HAI titer at D22	H1N1 HAI titer at D43	
					Boosted mice	Non-boosted mice
0.1 μg HA	3.467	4.131	4.063	57	95	80
0.4 μg HA	3.816	4.527	4.313	73	226	80
1.6 μg HA	4.069	4.831	4.750	147	1280	135
6.3 μg HA	4.534	5.298	4.947	320	1522	320
0.1 μg HA + emulsion	4.200	5.015	4.807	207	2153	538
0.4 μg HA + emulsion	4.612	5.482	5.001	453	2560	640

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Vaccine composition	H1N1 ELISA titer at D43		H3N2 ELISA titer at D43		B ELISA titer at D43	
	Boosted mice	Non-boosted mice	Boosted mice	Non-boosted mice	Boosted mice	Non-boosted mice
0.1 μg HA	3.833	3.425	4.782	4.174	4.479	3.911
0.4 μg HA	4.385	3.599	5.250	4.354	5.053	4.079
1.6 μg HA	4.906	3.876	5.526	4.779	5.458	4.487
6.3 μg HA	5.287	4.176	6.073	5.033	5.678	4.773
0.1 μg HA + emulsion	5.210	4.523	5.990	5.264	5.723	4.943
0.4 μg HA + emulsion	5.394	4.790	6.171	5.640	5.962	5.144

These results show that, by virtue of the emulsion according to the invention, even with low doses of antigens, very substantial humoral responses were obtained. Thus,

it can be noted that the best results are those obtained with a dose of 0.4 μg of HA of each of the viral strains and an emulsion according to the invention; these results are, very surprisingly, much better than those obtained by using a dose of 6.3 μg of HA alone. In addition, it is noted that, even in the individuals not given a booster dose, the immune system continues to induce antibodies, whereas this is not the case for the individuals given the non-adjuvanted vaccine antigens.

Example 8: Trivalent vaccine composition against the flu

30 μl immunization doses were prepared from the concentrated emulsion of example 1 and a vaccine composition comprising the 3 viral strains of the 2004 campaign (the A/New Caledonia (H1N1) strain, the A/Wyoming (H3N2) strain and the B/Jiangsu strain), these immunization doses having the following compositions:

- 0.1 μg of HA of each of the viral strains in PBS buffer,
- 0.4 μg of HA of each of the viral strains in PBS buffer,
- 1.6 μg of HA of each of the viral strains in PBS buffer,
- 6.3 μg of HA of each of the viral strains in PBS buffer,
- 0.1 μg of HA; 0.75 mg of squalene; 0.11 mg of Dehymuls™ SMO; 0.143 mg of Eumulgin™ B1 and 0.138 mg of mannitol in PBS buffer at pH 7.4,
- 0.4 μg of HA; 0.75 mg of squalene; 0.11 mg of Dehymuls™ SMO; 0.143 mg of Eumulgin™ B1 and 0.138 mg of mannitol in PBS buffer at pH 7.4.

Six groups of eight 8-week-old female C57BL/6J mice were provided and were administered, on D0 intradermally (internal face of the ear), with a dose of 30 μl of one of the compositions indicated above (1 composition per group).

Blood samples were taken on D23 in order to determine the amounts of antibodies induced.

The antibody titers were assayed by ELISA and by HAI for the antibodies induced with respect to all the strains administered: H1N1, H3N2 and B.

The results obtained have been summarized in the table below, in which the titers expressed are the means obtained for each group of mice.

Composition	ELISA H1N1	ELISA H3N2	ELISA B	HAI H1N1	HAI H3N2	HAI B
0.1 μg HA	2.924	3.506	3.920	26	174	95
0.4 μg HA	3.673	4.227	4.431	135	269	147
1.6 μg HA	3.948	4.593	4.786	160	381	190
6.3 μg HA	4.446	5.106	5.190	587	1174	453
0.1 μg HA + emulsion	4.456	5.192	5.064	349	1974	320
0.4 μg HA + emulsion	4.659	5.337	5.174	761	2348	494

Again, the results obtained show the great advantage of the emulsion according to the invention, by virtue of which it is possible to very substantially reduce the amounts of antigens present. Specifically, it can be considered, overall, that with only 0.1 μg of HA adjuvanted with the emulsion according to the invention, results are obtained that are as good as with an amount of 6.3 μg of HA.

Example 9: Trivalent vaccine composition against the flu comprising an emulsion according to the invention or according to the prior art

30 μl immunization doses were prepared from the concentrated emulsion of example 1 and a vaccine composition comprising the 3 viral strains of the 2004 campaign (the A/New Caledonia (H1N1) strain, the A/Wyoming (H3N2) strain and the B/Jiangsu strain), these immunization doses having the following compositions:

- 0.3 μg of HA of each of the viral strains in PBS buffer,
- 6.3 μg of HA of each of the viral strains in PBS buffer,
- 0.3 μg of HA; 0.21 mg of squalene; 0.031 mg of Dehymuls™ SMO; 0.040 mg of Eumulgin™ B1 and 0.039 mg of mannitol in PBS buffer at pH 7.4 (emulsion at 0.7%),
- 0.3 μg of HA; 0.75 mg of squalene; 0.11 mg of Dehymuls™ SMO; 0.143 mg of Eumulgin™ B1 and 0.138 mg of mannitol in PBS buffer at pH 7.4 (emulsion at 2.5%),

0.3 μg of HA; 0.645 mg of squalene; 0.075 mg of Tween™ 80; 0.075 mg of Span™ 85 (emulsion according to the prior art obtained by microfluidization).

Five groups of eight 8-week-old female BALB/c mice were provided and were administered, on D0 intradermally (internal face of the ear), with a dose of 30 μl of one of the compositions indicated above (1 composition per group).

To evaluate the amount of antibodies induced, blood samples were taken on D21 and the activity against the A/H1N1 strain, the A/H3N2 strain and the B strain was determined on said blood samples by HAI (hemagglutination inhibition).

The results obtained for each group of mice are represented in the table below.

Composition tested	HAI against H1N1	HAI against H3N2	HAI against B
0.3 μg HA	26	174	8
6.3 μg HA	247	905	73
0.3 μg HA + invention emulsion at 0.7%	95	640	37
0.3 μg HA + invention emulsion at 2.5%	269	1974	73
0.3 μg HA + prior art emulsion	135	987	57

These results show that, with an emulsion obtained according to the invention by virtue of a very simple preparation process consisting of phase inversion by means of a change in temperature, an adjuvant was obtained which is as good as, and even slightly better than, the emulsion of the prior art obtained using very high shear rates.

Example 10: Trivalent vaccine composition against the flu comprising an emulsion according to the invention at various concentrations

30 μl immunization doses were prepared from the concentrated emulsion of example 1 and a vaccine composition comprising the 3 viral strains of the 2004 campaign (the

A/New Caledonia (H1N1) strain, the A/Wyoming (H3N2) strain and the B/Jiangsu strain), these immunization doses having the following compositions:

- 0.3 µg of HA of each of the viral strains in PBS buffer,
- 6.3 µg of HA of each of the viral strains in PBS buffer,
- 0.3 µg of HA of each of the viral strains; 0.12 mg of squalene; 0.018 mg of Dehymuls™ SMO; 0.023 mg of Eumulgin™ B1 and 0.022 mg of mannitol in PBS buffer at pH 7.4 (emulsion at 0.4%),
- 0.3 µg of HA of each of the viral strains; 0.299 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 (emulsion at 1%),
- 0.3 µg of 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 in PBS buffer at pH 7.4 (emulsion at 2.5%).

Five groups of eight 8-week-old female BALB/c mice were provided and were administered, on D0 intradermally (internal face of the ear), with a dose of 30 µl of one of the compositions indicated above (1 composition per group).

In order to evaluate the amount of antibodies induced, blood samples were taken at D21 and the anti-H1N1 antibodies, the anti-H3N2 antibodies and the anti-B antibodies were determined on these blood samples by ELISA, and the activity against the A/H1N1 strain, the A/H3N2 strain and the B strain was determined by HAI (hemagglutination inhibition).

The results obtained are represented in the table below in the form of means for each of the groups; the ELISA results are expressed in $\log_{10}$ of arbitrary ELISA units and the HAI results are the mean arithmetic titers of the inverses of dilutions.

Composition tested	Anti-H1N1		Anti-H3N2		Anti-B	
	ELISA	HAI	ELISA	HAI	ELISA	HAI
0.3 μg HA	3.864	67	4.594	320	4.406	31
6.3 μg HA	4.478	269	5.041	1660	5.053	135
0.3 μg HA + invention emulsion at 0.4%	4.053	108	4.827	525	4.545	54
0.3 μg HA + invention emulsion at 1%	4.312	269	5.074	1174	4.733	123
0.3 μg HA + invention emulsion at 2.5%	4.425	293	5.200	1974	4.840	123

These results confirm once again that, whatever the strain evaluated, the emulsion according to the invention made it possible, with a very low dose of antigens, to obtain a very substantial immune system response.

Example 11: Preparation of a lyophilizable composition

The process was carried out as in example 1, but using water instead of the buffer; the emulsion obtained was subsequently diluted with an aqueous solution comprising mannitol, sucrose and dodecylmaltoside, in order to obtain an emulsion whose final composition was as follows:

- 5% of squalene,
- 0.95% of polyoxyethylene cetostearyl ether,
- 0.75% of sorbitan monooleate
- 3% of mannitol,
- 2% of dodecylmaltoside,
- 6% of sucrose.

This emulsion was lyophilized and conserved at 4°C for 3 months; then, after reconstitution, it was noted that its properties were conserved, in particular its monodisperse emulsion qualities, with d50 and d90 values close to those measured before lyophilization.

This emulsion was able to be diluted 50/50 with a solution comprising vaccine antigens in order to obtain a vaccine composition.

Example 12: Comparison of the adjuvant effect of the emulsion according to the invention and of a surfactant present in the emulsion

The intention was to evaluate the adjuvant activity of the emulsion according to the invention, compared with that of the surfactant Eumulgin™ B1 which is present in the emulsion.

For this, a test was carried out on mice using flu antigens.

To this end, flu virus antigens were available, obtained according to the process described in the examples of application WO 96/05294, with the exception of the fact that the viral strain used was the A/New Caledonia H1N1 strain. A vaccine composition comprising the 3 viral strains of the 2004 campaign (the A/New Caledonia (H1N1) strain, the A/Wyoming (H3N2) strain and the B/Jiangsu strain) was also available.

100 µl immunization doses were prepared from the concentrated emulsion obtained according to the invention and described in example 1, from Eumulgin™ B1, and from the flu virus antigen compositions, these immunization doses having the following compositions:

- 1 µg of HA of the H1N1 strain in PBS buffer at pH 7.4;
- 5 µg of HA of the H1N1 strain in PBS buffer at pH 7.4;
- 1 µg of HA of the H1N1 strain; 2.5 mg of squalene; 0.37 mg of Dehymuls™ SMO; 0.48 mg of Eumulgin™ B1 and 0.46 mg of mannitol in PBS buffer at pH 7.4 ;
- 1 µg of HA of the H1N1 strain and 0.48 mg of Eumulgin™ B1 in PBS buffer at pH 7.4 ;
- 0.33 µg of HA of each of the viral stains in PBS buffer at pH 7.4;
- 1.66 µg of HA of each of the viral stains in PBS buffer at pH 7.4;
- 0.33 µg of HA of each of the viral stains; 2.5mg of squalene; 0.37 mg of Dehymuls™ SMO; 0.48 mg of Eumulgin™ B1 and 0.46 mg of mannitol in PBS buffer at pH 7.4;
- 0.33 µg of HA of each of the viral stains and 0.48 mg of Eumulgin™ B1 in PBS buffer at pH 7.4.

Eight groups of 8 female BALB/c mice were provided and were immunized by means of a single intramuscular injection on D0. Blood samples were taken on D21 and D35 in order to evaluate by ELISA assay their content of total antibodies induced against the A/H1N1 flu strain or against each of the strains of the trivalent vaccine. The antibody titers, indicated in the table below, are expressed as the log10 value of arbitrary ELISA units, with a detection threshold of 1.3 log10.

	Titers at D21			Titers at D35		
Immunization dose compositions	H1N1	H3N2	B	H1N1	H3N2	B
H1N1 at 1 μg	2.745			2.848		
H1N1 at 5 μg	3.057			3.139		
H1N1 at 1 μg + invention emulsion	4.002			4.128		
H1N1 at 1 μg + Eumulgin™ B1	3.019			3.119		
Trivalent vaccine at 1 μg of total HA	3.111	3.718	3.706	3.218	4.147	3.935
Trivalent vaccine at 5 μg of total HA	3.692	4.386	3.975	3.776	4.632	4.225
Trivalent vaccine at 1 μg of total HA + invention emulsion	4.252	5.002	4.596	4.186	5.214	4.897
Trivalent vaccine at 1 μg of total HA + Eumulgin™ B1	3.146	3.712	3.950	3.241	4.177	4.242

The results obtained in this test confirm those already obtained in previous tests, namely that the emulsion according to the invention makes it possible to greatly reduce the dose of antigens for the same immune system response; a better response was in fact obtained using the emulsion according to the invention and only 1 μg of HA rather than using a dose of 5 μg of HA without adjuvant.

It is also observed that the surfactant used does not really exhibit any adjuvant effect when it is used alone, whereas the emulsion according to the invention itself produces a highly adjuvant effect with respect to all the strains tested.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. An oil-in-water adjuvant emulsion comprising at least:

- squalene,
- an aqueous solvent,
- a hydrophilic nonionic surfactant which is a polyoxyethylene alkyl ether,
- a hydrophobic nonionic surfactant,

which is thermoreversible and wherein 90% of the population by volume of the oil drops has a size less than 200 nm, and wherein the emulsion is obtained by a temperature-variation phase-inversion process.

2. The emulsion as claimed in claim 1, wherein 90% of the population by volume of the oil drops has a size less than 160 nm.

3. The emulsion as claimed in claim 1 or 2, wherein 90% of the population by volume of the oil drops has a size less than 150 nm.

4. The emulsion as claimed in any one of claims 1 to 3, wherein 50% of the population by volume of the oil drops has a size less than 100 nm.

5. The emulsion as claimed in any one of claims 1 to 4, wherein 50% of the population by volume of the oil drops has a size less than 90 nm.

6. The emulsion as claimed in any one of claims 1 to 5, which also comprises at least one alditol.

7. The emulsion as claimed in claim 1, wherein the hydrophobic nonionic surfactant comprises a sorbitan ester or a mannide ester.

8. The emulsion as claimed in any one of claims 1 to 7, wherein the polyoxyethylene alkyl ether is polyoxyethylene(12) cetostearyl ether.

9. The emulsion as claimed in any one of claims 1 to 8, wherein the alditol is chosen from glycerol, erythritol, xylitol, sorbitol and mannitol.

10. The emulsion as claimed in any one of claims 1 to 9, wherein the hydrophobic nonionic surfactant is sorbitan monooleate.

11. The emulsion as claimed in any one of claims 1 to 10, wherein the amount of squalene is between 5 and 45%.

12. The emulsion as claimed in any one of claims 1 to 11, wherein the amount of polyoxyethylene alkyl ether-based surfactant is between 0.9 and 9%.

13. The emulsion as claimed in any one of claims 1 to 12, wherein the amount of hydrophobic nonionic surfactant is between 0.7 and 7%.

14. The emulsion as claimed in any one of claims 1 to 13, which comprises:

- 32.5% of squalene,
- 6.18% of polyoxyethylene(12) cetostearyl ether,
- 4.82% of sorbitan monooleate,
- 6% of mannitol.

15. The emulsion as claimed in any one of claims 1 to 14, which also comprises alkylpolyglycoside.

16. The emulsion as claimed in any one of claims 1 to 15, which also comprises a cryoprotective agent.

17. The use of an emulsion as claimed in any one of claims 1 to 16 for preparing an immunogenic composition intended to be administered intramuscularly.

18. The use of an emulsion as claimed in any one of claims 1 to 16, for preparing an immunogenic composition intended to be administered intradermally.

19. The use of an emulsion as claimed in any one of claims 1 to 16, for preparing an immunogenic composition intended to be administered subcutaneously.

20. The use of an emulsion as claimed in any one of claims 1 to 16, for preparing an immunogenic composition intended to be administered against the flu.

21. The use of an emulsion as claimed in any one of claims 1 to 16, for preparing an immunogenic composition intended to be administered against AIDS.

22. The use of an emulsion as claimed in any one of claims 1 to 16, for preparing an immunogenic composition intended to be administered against human cytomegalovirus pathologies.

23. A process for preparing an immunogenic composition, according to which at least one vaccine antigen is mixed with an oil-in-water emulsion as claimed in any one of claims I to 16, wherein the oil-in-water emulsion is obtained by means of a temperature-variation phaseinversion process.

24. The process as claimed in claim 23, which comprises at least a step for preparing the oil-in-water emulsion by cooling a water-in-oil inverse emulsion, which comprises at least:

- squalene,
- an aqueous solvent,
- a hydrophilic nonionic surfactant which is polyoxyethylene alkyl ether,
- a hydrophobic nonionic surfactant,

wherein 90% of the population by volume of the oil drops in the emulsion have a size less than 200 nm.

25. The process as claimed in claim 24, wherein the water-in-oil inverse emulsion is obtained by mixing squalene, an aqueous solvent, a nonionic surfactant which is a polyoxyethylene alkyl ether, and a hydrophobic nonionic surfactant so as to obtain, first of all, an oil-in-water coarse emulsion, and this emulsion is then heated to at least the phaseinversion temperature so as to obtain the inverse emulsion.

26. The process as claimed in claim 24, according to which:

- firstly, an aqueous phase comprising an aqueous solvent and a surfactant

which is a polyoxyethylene alkyl ether and, secondly, an oily phase comprising squalene and a hydrophobic surfactant are heated, separately, to a temperature at least equal to the phaseinversion temperature, and then

- the 2 phases are mixed so as to obtain a water-in-oil inverse emulsion.

27. The process as claimed in claim 24, according to which:

- firstly, an aqueous phase comprising an aqueous solvent and a surfactant

which is a polyoxyethylene alkyl ether and, secondly, an oily phase comprising squalene and a hydrophonic surfactant are heated, separately, to a temperature below the phase-inversion temperature of the emulsion,

- the 2 phases are then mixed so as to obtain an oil-in-water emulsion,
- the oil-in-water emulsion obtained is then heated to a temperature at least

equal to the phase-inversion temperature so as to obtain a water-in-oil inverse emulsion.

28. The process as claimed in any one of claims 23 to 27, wherein the phase-inversion temperature is between 45 and 80°C.

29. The process as claimed in claim 28, wherein the phase-inversion temperature is between 50 and 65°C.

30. The process as claimed in any one of claims 23 to 29, which also comprises at least one lyophilization step.

31. An immunogenic composition, which can be obtained by means of the process as claimed in any one of claims 23 to 30.

32. A method for prophylactic or therapeutic treatment of the flu, AIDS or human cytomegalovirus pathologies, comprising administering to a subject in need thereof an immunogenic composition comprising an emulsion as claimed in any one of claims 1 to 16.

33. The emulsion as claimed in claim 1, the use as claimed in any one of claims 17 to 22, the process as claimed in claim 23, the immunogenic composition according to claim 31, or the method as claimed in claim 32, substantially as hereinbefore described with reference to any one of the examples.