

(19)



(11) Publication number:

SG 182957 A1

(43) Publication date:

30.08.2012

(51) Int. Cl:

;

(12)

Patent Application

(21) Application number: **2012047478**

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(22) Date of filing: **27.06.2008**

(30) Priority: **US 60/937,515 27.06.2007**

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(54) **Title:**

LOW-ADDITIVE INFLUENZA VACCINES

(57) **Abstract:**

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LOW-ADDITIVE INFLUENZA VACCINES

TECHNICAL FIELD

This invention is in the field of vaccines for protecting against influenza virus infection, and in particular vaccines that contain low levels of pharmaceutical additives.

5 BACKGROUND ART

Various forms of influenza virus vaccine are currently available (*e.g.* see chapters 17 & 18 of reference 1). Vaccines are generally based either on live virus or on inactivated virus. Inactivated vaccines may be based on whole virions, 'split' virions, or on purified surface antigens.

10 In addition to their antigenic content, current influenza vaccines include various pharmaceutical additives and other contaminants, such as: anti-bacterial preservatives *e.g.* thimerosal; detergents *e.g.* CTAB, polysorbate 80, octoxynol 10, *etc.*; antibiotics *e.g.* neomycin, kanamycin; formaldehyde; and egg-derived materials including egg proteins (*e.g.* ovomucoid) and chicken DNA.

For the 2006/07 season, for instance, manufacturers' datasheets for the four inactivated vaccines used in the USA reveal the following information:

Vaccine	Preservative	Antibiotic(s)	Formaldehyde	Egg materials
Fluarix™	Thimerosal	Gentamycin	Yes	Yes
Fluvirin™	Thimerosal	No	No	Yes
Fluzone™	Optional thimerosal	No	Yes	Yes
FluLaval™	Thimerosal	No	Yes	Yes

15 Similar to the Fluvirin™ 2006-07 product, reference 2 prepared a vaccine that was free of formaldehyde, but contained thimerosal and egg products.

It is an object of the invention to provide further and improved influenza vaccines, and processes for their manufacture, which reduce or eliminate the amount and/or number of these pharmaceutical additives, thereby giving a purer vaccine product.

20 DISCLOSURE OF THE INVENTION

According to the invention, an influenza vaccine lacks at least three of: a mercurial preservative; an antibiotic; formaldehyde; and egg-derived materials. In some embodiments, a vaccine includes none of these four components.

25 Thus in one embodiment the invention provides a vaccine comprising an influenza virus antigen, wherein the vaccine contains no mercurial preservative, no antibiotic and no egg-derived materials.

Formaldehyde-free vaccines are preferred. Thus the invention provides a vaccine comprising an influenza virus antigen, wherein the vaccine contains no mercurial preservative, no antibiotic and no formaldehyde. The invention also provides a vaccine comprising an influenza virus antigen, wherein the vaccine contains no antibiotic, no formaldehyde and no egg-derived materials.

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5 Preferred vaccines also have a very low endotoxin content *e.g.* less than 0.1 IU/ml, and preferably less than 0.05 IU/ml. The international unit for endotoxin measurement is well known and can be calculated for a sample by, for instance, comparison to an international standard [3,4], such as the
10 *2nd International Standard* (Code 94/580 - IS) available from the NIBSC. Current vaccines prepared from virus grown in eggs have endotoxin levels in the region of 0.5-5 IU/ml.

The invention also provides a process for preparing an influenza virus antigen, comprising the steps of: (i) growing influenza virus in a cell culture system, in the absence of egg-derived materials and of
10 antibiotics; (ii) inactivating the influenza viruses grown in step (i), in the absence of formaldehyde; and (iii) preparing a vaccine antigen formulation from the inactivated influenza viruses, in the absence of thimerosal. The resulting antigen formulation may a bulk vaccine antigen that can be used to prepare monovalent or multivalent vaccines.

Antigen components

15 The invention uses influenza virus antigens prepared from influenza virions.

The virions are inactivated without using formaldehyde. Chemical means for inactivating a virus include treatment with an effective amount of one or more of the following agents: detergents, β -propiolactone, or UV light. Additional chemical means for inactivation include treatment with
20 methylene blue, psoralen, carboxyfullerene (C60) or a combination of any thereof. Other methods of viral inactivation are known in the art, such as for example binary ethylamine, acetyl ethyleneimine, or gamma irradiation.

Virions can be harvested from virus-containing fluids by various methods. For example, a purification process may involve zonal centrifugation using a linear sucrose gradient solution that includes detergent to disrupt the virions. Antigens may then be purified, after optional dilution, by
25 diafiltration.

Split virions are obtained by treating purified virions with detergents (*e.g.* ethyl ether, polysorbate 80, deoxycholate, tri-*N*-butyl phosphate, Triton X-100, Triton N101, cetyltrimethylammonium bromide, Tergitol NP9, *etc.*) to produce subvirion preparations, including the 'Tween-ether' splitting process. Methods of splitting influenza viruses are well known in the art *e.g.* see refs. 5-10, *etc.* Splitting of
30 the virus is typically carried out by disrupting or fragmenting whole virus, whether infectious or non-infectious, with a disrupting concentration of a splitting agent. The disruption results in a full or partial solubilisation of the virus proteins, altering the integrity of the virus. Preferred splitting agents are non-ionic and ionic (*e.g.* cationic) surfactants *e.g.* alkylglycosides, alkylthioglycosides, acyl sugars, sulphobetaines, betains, polyoxyethylenealkylethers, *N,N*-dialkyl-Glucamides, Hecameg,
35 alkylphenoxy-polyethoxyethanols, quaternary ammonium compounds, sarcosyl, CTABs (cetyl trimethyl ammonium bromides), tri-*N*-butyl phosphate, Cetavlon, myristyltrimethylammonium salts,

lipofectin, lipofectamine, and DOTMA, the octyl- or nonylphenoxy polyoxyethanols (*e.g.* the Triton surfactants, such as Triton X-100 or Triton N101), polyoxyethylene sorbitan esters (the Tween surfactants), polyoxyethylene ethers, polyoxyethylene esters, *etc.* One useful splitting procedure uses the consecutive effects of sodium deoxycholate and formaldehyde, and splitting can take place during initial virion purification (*e.g.* in a sucrose density gradient solution). Split virions can usefully be resuspended in sodium phosphate-buffered isotonic sodium chloride solution. s.

Purified surface antigen vaccines comprise the influenza surface antigens haemagglutinin and, typically, also neuraminidase. Processes for preparing these proteins in purified form are well known.

Influenza antigens can also be presented in the form of virosomes [11] (nucleic acid free viral-like liposomal particles), as in the INFLEXAL V™ and INVAVACT™ products.

The influenza virus may be attenuated. The influenza virus may be temperature-sensitive. The influenza virus may be cold-adapted. These three features are, however, associated more with live virus vaccines.

Human influenza virus strains for use in vaccines change from season to season. In the current inter-pandemic period, vaccines typically include two influenza A strains (H1N1 and H3N2) and one influenza B strain, and trivalent vaccines are typical. The invention may also use HA from pandemic strains (*i.e.* strains to which the vaccine recipient and the general human population are immunologically naïve), such as H2, H5, H7 or H9 subtype strains (in particular of influenza A virus), and influenza vaccines for pandemic strains may be monovalent or may be based on a normal trivalent vaccine supplemented by a pandemic strain. Depending on the season and on the nature of the antigen included in the vaccine, however, the invention may protect against one or more of HA subtypes H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15 or H16 (influenza A virus). The invention may protect against one or more of influenza A virus NA subtypes N1, N2, N3, N4, N5, N6, N7, N8 or N9.

As well as being suitable for immunizing against inter-pandemic strains, the compositions of the invention are particularly useful for immunizing against pandemic strains. The characteristics of an influenza strain that give it the potential to cause a pandemic outbreak are: (a) it contains a new hemagglutinin compared to the hemagglutinins in currently-circulating human strains, *i.e.* one that either has not been evident in the human population for over a decade (*e.g.* H2) or has not previously been seen at all in the human population (*e.g.* H5, H6 or H9, that have generally been found only in bird populations), and/or it contains a new neuraminidase compared to the neuraminidases in currently-circulating human strains, such that the human population will be immunologically naïve to the strain's hemagglutinin and/or neuraminidase; (b) it is capable of being transmitted horizontally in the human population; and (c) it is pathogenic to humans. A virus with H5 haemagglutinin type is preferred for immunizing against pandemic influenza, such as a H5N1 strain. Other possible strains include H5N3, H9N2, H2N2, H7N1 and H7N7, and any other emerging potentially pandemic strains.

Within the H5 subtype, a virus may fall into HA clade 1, HA clade 1', HA clade 2 or HA clade 3 [12], with clades 1 and 3 being particularly relevant.

5 Other strains whose antigens can usefully be included in the compositions are strains which are resistant to antiviral therapy (e.g. resistant to oseltamivir [13] and/or zanamivir), including resistant pandemic strains [14].

10 Compositions of the invention may include antigen(s) from one or more (e.g. 1, 2, 3, 4 or more) influenza virus strains, including influenza A virus and/or influenza B virus. Monovalent vaccines can be prepared, as can 2-valent, 3-valent, 4-valent, etc.. Where a vaccine includes more than one strain of influenza, the different strains are typically grown separately and are mixed after the viruses have been harvested and antigens have been prepared. Thus a process of the invention may include the step of mixing antigens from more than one influenza strain, and this process may be performed under non-refrigerated conditions. A trivalent vaccine is preferred, including antigens from two influenza A virus strains and one influenza B virus strain.

15 In some embodiments of the invention, the compositions may include antigen from a single influenza A strain. In some embodiments, the compositions may include antigen from two influenza A strains, provided that these two strains are not H1N1 and H3N2. In some embodiments, the compositions may include antigen from more than two influenza A strains.

20 The influenza virus may be a reassortant strain, and may have been obtained by reverse genetics techniques. Reverse genetics techniques [e.g. 15-19] allow influenza viruses with desired genome segments to be prepared *in vitro* using plasmids. Typically, it involves expressing (a) DNA molecules that encode desired viral RNA molecules e.g. from polII promoters, and (b) DNA molecules that encode viral proteins e.g. from polIII promoters, such that expression of both types of DNA in a cell leads to assembly of a complete intact infectious virion. The DNA preferably provides all of the viral RNA and proteins, but it is also possible to use a helper virus to provide some of the RNA and proteins. Plasmid-based methods using separate plasmids for producing each viral RNA are preferred [20-22], and these methods will also involve the use of plasmids to express all or some (e.g. just the PB1, PB2, PA and NP proteins) of the viral proteins, with up to 12 plasmids being used in some methods. To reduce the number of plasmids needed, a recent approach [23] combines a plurality of RNA polymerase I transcription cassettes (for viral RNA synthesis) on the same plasmid (e.g. sequences encoding 1, 2, 3, 4, 5, 6, 7 or all 8 influenza A vRNA segments), and a plurality of protein-coding regions with RNA polymerase II promoters on another plasmid (e.g. sequences encoding 1, 2, 3, 4, 5, 6, 7 or all 8 influenza A mRNA transcripts). Preferred aspects of the reference 23 method involve: (a) PB1, PB2 and PA mRNA-encoding regions on a single plasmid; and (b) all 8 vRNA-encoding segments on a single plasmid. Including the NA and HA segments on one plasmid and the six other segments on another plasmid can also facilitate matters.

35 As an alternative to using polII promoters to encode the viral RNA segments, it is possible to use bacteriophage polymerase promoters [24]. For instance, promoters for the SP6, T3 or T7

polymerases can conveniently be used. Because of the species-specificity of polI promoters, bacteriophage polymerase promoters can be more convenient for many cell types (*e.g.* MDCK), although a cell must also be transfected with a plasmid encoding the exogenous polymerase enzyme.

5 In other techniques it is possible to use dual polI and polIII promoters to simultaneously code for the viral RNAs and for expressible mRNAs from a single template [25,26].

Thus the virus, particularly an influenza A virus, may include one or more RNA segments from a A/PR/8/34 virus (typically 6 segments from A/PR/8/34, with the HA and N segments being from a vaccine strain, *i.e.* a 6:2 reassortant). It may also include one or more RNA segments from a A/WSN/33 virus, or from any other virus strain useful for generating reassortant viruses for vaccine
10 preparation. Typically, the invention protects against a strain that is capable of human-to-human transmission, and so the strain's genome will usually include at least one RNA segment that originated in a mammalian (*e.g.* in a human) influenza virus. It may include NS segment that originated in an avian influenza virus.

As mentioned above, the viruses used as the source of the antigens are generally grown on cell
15 culture, thereby avoiding contamination with components from embryonated hen eggs. Thus vaccines of the invention can be free from chicken DNA, as well as being free from egg proteins (such as ovalbumin and ovomucoid).

The cell substrate will typically be a cell line of mammalian origin. Suitable mammalian cells of origin include, but are not limited to, hamster, cattle, primate (including humans and monkeys) and
20 dog cells. Various cell types may be used, such as kidney cells, fibroblasts, retinal cells, lung cells, *etc.* Examples of suitable hamster cells are the cell lines having the names BHK21 or HKCC. Suitable monkey cells are *e.g.* African green monkey cells, such as kidney cells as in the Vero cell line. Suitable dog cells are *e.g.* kidney cells, as in the MDCK cell line. Thus suitable cell lines include, but are not limited to: MDCK; CHO; 293T; BHK; Vero; MRC-5; PER.C6; WI-38; *etc.*

25 Preferred mammalian cell lines for growing influenza viruses include: MDCK cells [27-30], derived from Madin Darby canine kidney; Vero cells [31-33], derived from African green monkey (*Cercopithecus aethiops*) kidney; or PER.C6 cells [34], derived from human embryonic retinoblasts. These cell lines are widely available *e.g.* from the American Type Cell Culture (ATCC) collection [35], from the Coriell Cell Repositories [36], or from the European Collection of Cell Cultures
30 (ECACC). For example, the ATCC supplies various different Vero cells under catalog numbers CCL-81, CCL-81.2, CRL-1586 and CRL-1587, and it supplies MDCK cells under catalog number CCL-34. PER.C6 is available from the ECACC under deposit number 96022940. As an alternative to mammalian cell lines, virus can be grown on avian cell lines [*e.g.* refs. 37-39], including avian embryonic stem cells [37,40] and cell lines derived from ducks (*e.g.* duck retina), or from hens.
35 Suitable avian embryonic stem cells, include the EBx cell line derived from chicken embryonic stem cells, EB45, EB14, and EB14-074 [41]. Chicken embryo fibroblasts (CEF), can also be used, *etc.*

The most preferred cell lines for growing influenza viruses are MDCK cell lines. The original MDCK cell line is available from the ATCC as CCL-34, but derivatives of this cell line may also be used. For instance, reference 27 discloses a MDCK cell line that was adapted for growth in suspension culture ('MDCK 33016', deposited as DSM ACC 2219). Similarly, reference 42
5 discloses a MDCK-derived cell line that grows in suspension in serum-free culture ('B-702', deposited as FERM BP-7449). Reference 43 discloses non-tumorigenic MDCK cells, including 'MDCK-S' (ATCC PTA-6500), 'MDCK-SF101' (ATCC PTA-6501), 'MDCK-SF102' (ATCC PTA-6502) and 'MDCK-SF103' (PTA-6503). Reference 44 discloses MDCK cell lines with high susceptibility to infection, including 'MDCK.5F1' cells (ATCC CRL-12042). Any of these MDCK
10 cell lines can be used.

The culture for cell growth, and also the viral inoculum used to start the culture, will preferably be free from (*i.e.* will have been tested for and given a negative result for contamination by) herpes simplex virus, respiratory syncytial virus, parainfluenza virus 3, SARS coronavirus, adenovirus, rhinovirus, reoviruses, polyomaviruses, birnaviruses, circoviruses, and/or parvoviruses [45]. Absence
15 of herpes simplex viruses is particularly preferred.

Virus may be grown on cells in suspension [27,46,47] or in adherent culture. In one embodiment, the cells may be adapted for growth in suspension. One suitable MDCK cell line that is adapted for growth in suspension culture is MDCK 33016 (deposited as DSM ACC 2219). As an alternative, microcarrier culture can be used.

20 Cell lines supporting influenza virus replication are preferably grown in serum-free culture media and/or protein free media. A medium is referred to as a serum-free medium in the context of the present invention in which there are no additives from serum of human or animal origin. Protein-free is understood to mean cultures in which multiplication of the cells occurs with exclusion of proteins, growth factors, other protein additives and non-serum proteins, but can optionally include proteins
25 such as trypsin or other proteases that may be necessary for viral growth. The cells growing in such cultures naturally contain proteins themselves.

Cell lines supporting influenza virus replication are preferably grown below 37°C [48] (*e.g.* 30-36°C) during viral replication.

The method for propagating virus in cultured cells generally includes the steps of inoculating the
30 cultured cells with the strain to be cultured, cultivating the infected cells for a desired time period for virus propagation, such as for example as determined by virus titer or antigen expression (*e.g.* between 24 and 168 hours after inoculation) and collecting the propagated virus. The cultured cells are inoculated with a virus (measured by PFU or TCID₅₀) to cell ratio of 1:500 to 1:1, preferably 1:100 to 1:5, more preferably 1:50 to 1:10. The virus is added to a suspension of the cells or is
35 applied to a monolayer of the cells, and the virus is absorbed on the cells for at least 60 minutes but usually less than 300 minutes, preferably between 90 and 240 minutes at 25°C to 40°C, preferably 28°C to 37°C. The infected cell culture (*e.g.* monolayers) may be removed either by freeze-thawing

or by enzymatic action to increase the viral content of the harvested culture supernatants. The harvested fluids are then either inactivated or stored frozen. Cultured cells may be infected at a multiplicity of infection ("m.o.i.") of about 0.0001 to 10, preferably 0.002 to 5, more preferably to 0.001 to 2. Still more preferably, the cells are infected at a m.o.i of about 0.01. Infected cells may be harvested 30 to 60 hours post infection. Preferably, the cells are harvested 34 to 48 hours post infection. Still more preferably, the cells are harvested 38 to 40 hours post infection. Proteases (typically trypsin) are generally added during cell culture to allow viral release, and the proteases can be added at any suitable stage during the culture. According to the invention, antibiotics can be avoided during the culture.

10. Influenza vaccines are currently standardised by reference to HA levels, typically measured by SRID. Existing vaccines typically contain about 15µg of HA per strain, although lower doses can be used (e.g. when using an adjuvant). Fractional doses such as ½ (i.e. 7.5µg HA per strain), ¼ and 1/8 have been used [62,63], as have higher doses (e.g. 3x or 9x doses [49,50]). Thus vaccines may include between 0.1 and 150µg of HA per influenza strain, preferably between 0.1 and 50µg e.g. 0.1-20µg, 0.1-15µg, 0.1-10µg, 0.1-7.5µg, 0.5-5µg, etc. Particular doses include e.g. about 90, about 45, about 30, about 15, about 10, about 7.5, about 5, about 3.8, about 1.9, about 1.5, etc. per strain. The components of the vaccines, kits and processes of the invention (e.g. their volumes and concentrations) may be selected to provide these antigen doses in final products.

HA used with the invention may be a natural HA as found in a virus, or may have been modified. For instance, it is known to modify HA to remove determinants (e.g. hyper-basic regions, such as around the cleavage site between HA1 and HA2).

As well as including haemagglutinin, compositions of the invention may include further influenza virus proteins. For instance, they will typically include neuraminidase glycoprotein. They may also include a matrix protein, such as M1 and/or M2 (or a fragment thereof), and/or nucleoprotein.

25 *Host cell DNA*

Where virus has been grown on a cell line then it is standard practice to minimize the amount of residual cell line DNA in the final vaccine, in order to minimize any oncogenic activity of the DNA. Thus the composition preferably contains less than 10ng (preferably less than 1ng, and more preferably less than 100pg) of residual host cell DNA per dose, although trace amounts of host cell DNA may be present. In general, the host cell DNA that it is desirable to exclude from compositions of the invention is DNA that is longer than 100bp.

Measurement of residual host cell DNA is now a routine regulatory requirement for biologicals and is within the normal capabilities of the skilled person. The assay used to measure DNA will typically be a validated assay [51,52]. The performance characteristics of a validated assay can be described in mathematical and quantifiable terms, and its possible sources of error will have been identified. The assay will generally have been tested for characteristics such as accuracy, precision, specificity. Once

an assay has been calibrated (*e.g.* against known standard quantities of host cell DNA) and tested then quantitative DNA measurements can be routinely performed. Three principle techniques for DNA quantification can be used: hybridization methods, such as Southern blots or slot blots [53]; immunoassay methods, such as the Threshold™ System [54]; and quantitative PCR [55]. These methods are all familiar to the skilled person, although the precise characteristics of each method may depend on the host cell in question *e.g.* the choice of probes for hybridization, the choice of primers and/or probes for amplification, *etc.* The Threshold™ system from *Molecular Devices* is a quantitative assay for picogram levels of total DNA, and has been used for monitoring levels of contaminating DNA in biopharmaceuticals [54]. A typical assay involves non-sequence-specific formation of a reaction complex between a biotinylated ssDNA binding protein, a urease-conjugated anti-ssDNA antibody, and DNA. All assay components are included in the complete Total DNA Assay Kit available from the manufacturer. Various commercial manufacturers offer quantitative PCR assays for detecting residual host cell DNA *e.g.* AppTec™ Laboratory Services, BioReliance™, Althea Technologies, *etc.* A comparison of a chemiluminescent hybridisation assay and the total DNA Threshold™ system for measuring host cell DNA contamination of a human viral vaccine can be found in reference 56.

Contaminating DNA can be removed during vaccine preparation using standard purification procedures *e.g.* chromatography, *etc.* Removal of residual host cell DNA can be enhanced by nuclease treatment *e.g.* by using a DNase. A convenient method for reducing host cell DNA contamination is disclosed in references 57 & 58, involving a two-step treatment, first using a DNase (*e.g.* Benzonase), which may be used during viral growth, and then a cationic detergent (*e.g.* CTAB), which may be used during virion disruption. Treatment with an alkylating agent, such as β -propiolactone, can also be used to remove host cell DNA, and advantageously may also be used to inactivate virions [59] while avoiding use of formaldehyde.

Vaccines containing <10ng (*e.g.* <1ng, <100pg) host cell DNA per 15 μ g of haemagglutinin are preferred, as are vaccines containing <10ng (*e.g.* <1ng, <100pg) host cell DNA per 0.25ml volume. Vaccines containing <10ng (*e.g.* <1ng, <100pg) host cell DNA per 50 μ g of haemagglutinin are more preferred, as are vaccines containing <10ng (*e.g.* <1ng, <100pg) host cell DNA per 0.5ml volume.

Adjuvants

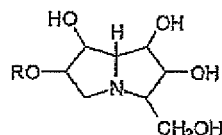
Compositions of the invention may advantageously include an adjuvant, which can function to enhance the immune responses (humoral and/or cellular) elicited in a patient who receives the composition. The use of adjuvants with influenza vaccines has been described before. In references 60 & 61, aluminum hydroxide was used, and in reference 62, a mixture of aluminum hydroxide and aluminum phosphate was used. Reference 63 also described the use of aluminum salt adjuvants. The FLUAD™ product from Chiron Vaccines includes an oil-in-water emulsion.

Adjuvants that can be used with the invention include, but are not limited to:

- A mineral-containing composition, including calcium salts and aluminum salts (or mixtures thereof). Calcium salts include calcium phosphate (*e.g.* the “CAP” particles disclosed in ref. 64). Aluminum salts include hydroxides, phosphates, sulfates, *etc.*, with the salts taking any suitable form (*e.g.* gel, crystalline, amorphous, *etc.*). Adsorption to these salts is preferred. The mineral containing compositions may also be formulated as a particle of metal salt [65]. Aluminum salt adjuvants are described in more detail below.
- Cytokine-inducing agents (see in more detail below).
- Saponins [chapter 22 of ref. 101], which are a heterologous group of sterol glycosides and triterpenoid glycosides that are found in the bark, leaves, stems, roots and even flowers of a wide range of plant species. Saponin from the bark of the *Quillaja saponaria* Molina tree have been widely studied as adjuvants. Saponin can also be commercially obtained from *Smilax ornata* (sarsapilla), *Gypsophilla paniculata* (brides veil), and *Saponaria officianalis* (soap root). Saponin adjuvant formulations include purified formulations, such as QS21, as well as lipid formulations, such as ISCOMs. QS21 is marketed as Stimulon™. Saponin compositions have been purified using HPLC and RP-HPLC. Specific purified fractions using these techniques have been identified, including QS7, QS17, QS18, QS21, QH-A, QH-B and QH-C. Preferably, the saponin is QS21. A method of production of QS21 is disclosed in ref. 66. It is possible to use fraction A of Quil A together with at least one other adjuvant [67]. Saponin formulations may also comprise a sterol, such as cholesterol [68]. Combinations of saponins and cholesterol can be used to form unique particles called immunostimulating complexes (ISCOMs) [chapter 23 of ref. 101]. ISCOMs typically also include a phospholipid such as phosphatidylethanolamine or phosphatidylcholine. Any known saponin can be used in ISCOMs. Preferably, the ISCOM includes one or more of QuilA, QHA & QHC. ISCOMs are further described in refs. 68-70. Optionally, the ISCOMS may be devoid of additional detergent [71]. It is possible to use a mixture of at least two ISCOM complexes, each complex comprising essentially one saponin fraction, where the complexes are ISCOM complexes or ISCOM matrix complexes [72]. A review of the development of saponin based adjuvants can be found in refs. 73 & 74.
- Fatty adjuvants (see in more detail below).
- Bacterial ADP-ribosylating toxins (*e.g.* the *E.coli* heat labile enterotoxin “LT”, cholera toxin “CT”, or pertussis toxin “PT”) and detoxified derivatives thereof, such as the mutant toxins known as LT-K63 and LT-R72 [75]. The use of detoxified ADP-ribosylating toxins as mucosal adjuvants is described in ref. 76 and as parenteral adjuvants in ref. 77.
- Bioadhesives and mucoadhesives, such as esterified hyaluronic acid microspheres [78] or chitosan and its derivatives [79].
- Microparticles (*i.e.* a particle of ~100nm to ~150µm in diameter, more preferably ~200nm to ~30µm in diameter, or ~500nm to ~10µm in diameter) formed from materials that are

biodegradable and non-toxic (e.g. a poly(α -hydroxy acid), a polyhydroxybutyric acid, a polyorthoester, a polyanhydride, a polycaprolactone, etc.), with poly(lactide-co-glycolide) being preferred, optionally treated to have a negatively-charged surface (e.g. with SDS) or a positively-charged surface (e.g. with a cationic detergent, such as CTAB).

- 5
- Liposomes (Chapters 13 & 14 of ref. 101). Examples of liposome formulations suitable for use as adjuvants are described in refs. 80-82.
 - Oil-in-water emulsions (see in more detail below).
 - Polyoxyethylene ethers and polyoxyethylene esters [83]. Such formulations further include polyoxyethylene sorbitan ester surfactants in combination with an octoxynol [84] as well as
- 10
- polyoxyethylene alkyl ethers or ester surfactants in combination with at least one additional non-ionic surfactant such as an octoxynol [85]. Preferred polyoxyethylene ethers are selected from the following group: polyoxyethylene-9-lauryl ether (laureth 9), polyoxyethylene-9-stearyl ether, polyoxyethylene-8-stearyl ether, polyoxyethylene-4-lauryl ether, polyoxyethylene-35-lauryl ether, and polyoxyethylene-23-lauryl ether.
- 15
- Muramyl peptides, such as N-acetylmuramyl-L-threonyl-D-isoglutamine ("thr-MDP"), N-acetyl-normuramyl-L-alanyl-D-isoglutamine (nor-MDP), N-acetylglucosaminyl-N-acetylmuramyl-L-Al-D-isoglu-L-Ala-dipalmitoxy propylamide ("DTP-DPP", or "Theramide™), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine ("MTP-PE").
- 20
- An outer membrane protein proteosome preparation prepared from a first Gram-negative bacterium in combination with a liposaccharide preparation derived from a second Gram-negative bacterium, wherein the outer membrane protein proteosome and liposaccharide preparations form a stable non-covalent adjuvant complex. Such complexes include "IVX-908", a complex comprised of *Neisseria meningitidis* outer membrane and lipopolysaccharides. They have been used as adjuvants for influenza vaccines [86].
- 25
- Methyl inosine 5'-monophosphate ("MIMP") [87].
 - A polyhydroxylated pyrrolizidine compound [88], such as one having formula:



30

where R is selected from the group comprising hydrogen, straight or branched, unsubstituted or substituted, saturated or unsaturated acyl, alkyl (e.g. cycloalkyl), alkenyl, alkynyl and aryl groups, or a pharmaceutically acceptable salt or derivative thereof. Examples include, but are not limited to: casuarine, casuarine-6- α -D-glucopyranose, 3-*epi*-casuarine, 7-*epi*-casuarine, 3,7-*diepi*-casuarine, etc.

- A gamma inulin [89] or derivative thereof, such as algammulin.

- A CD1d ligand, such as a α -glycosylceramide *e.g.* α -galactosylceramide.

These and other adjuvant-active substances are discussed in more detail in references 101 & 102.

Compositions may include two or more of said adjuvants. For example, they may advantageously include both an oil-in-water emulsion and a cytokine-inducing agent, as this combination improves the cytokine responses elicited by influenza vaccines, such as the interferon- γ response, with the improvement being much greater than seen when either the emulsion or the agent is used on its own.

Antigens and adjuvants in a composition will typically be in admixture.

Where a vaccine includes an adjuvant, it may be prepared extemporaneously, at the time of delivery. Thus the invention provides kits including the antigen and adjuvant components ready for mixing. The kit allows the adjuvant and the antigen to be kept separately until the time of use. The components are physically separate from each other within the kit, and this separation can be achieved in various ways. For instance, the two components may be in two separate containers, such as vials. The contents of the two vials can then be mixed *e.g.* by removing the contents of one vial and adding them to the other vial, or by separately removing the contents of both vials and mixing them in a third container. In a preferred arrangement, one of the kit components is in a syringe and the other is in a container such as a vial. The syringe can be used (*e.g.* with a needle) to insert its contents into the second container for mixing, and the mixture can then be withdrawn into the syringe. The mixed contents of the syringe can then be administered to a patient, typically through a new sterile needle. Packing one component in a syringe eliminates the need for using a separate syringe for patient administration. In another preferred arrangement, the two kit components are held together but separately in the same syringe *e.g.* a dual-chamber syringe, such as those disclosed in references 90-97 *etc.* When the syringe is actuated (*e.g.* during administration to a patient) then the contents of the two chambers are mixed. This arrangement avoids the need for a separate mixing step at the time of use.

25 *Oil-in-water emulsion adjuvants*

Oil-in-water emulsions have been found to be particularly suitable for use in adjuvanting influenza virus vaccines. Various such emulsions are known, and they typically include at least one oil and at least one surfactant, with the oil(s) and surfactant(s) being biodegradable (metabolisable) and biocompatible. The oil droplets in the emulsion are generally less than 5 μ m in diameter, and may even have a sub-micron diameter, with these small sizes being achieved with a microfluidiser to provide stable emulsions. Droplets with a size less than 220nm are preferred as they can be subjected to filter sterilization.

The invention can be used with oils such as those from an animal (such as fish) or vegetable source. Sources for vegetable oils include nuts, seeds and grains. Peanut oil, soybean oil, coconut oil, and olive oil, the most commonly available, exemplify the nut oils. Jojoba oil can be used *e.g.* obtained from the jojoba bean. Seed oils include safflower oil, cottonseed oil, sunflower seed oil, sesame seed

oil and the like. In the grain group, corn oil is the most readily available, but the oil of other cereal grains such as wheat, oats, rye, rice, teff, triticale and the like may also be used. 6-10 carbon fatty acid esters of glycerol and 1,2-propanediol, while not occurring naturally in seed oils, may be prepared by hydrolysis, separation and esterification of the appropriate materials starting from the nut and seed oils. Fats and oils from mammalian milk are metabolizable and may therefore be used in the practice of this invention. The procedures for separation, purification, saponification and other means necessary for obtaining pure oils from animal sources are well known in the art. Most fish contain metabolizable oils which may be readily recovered. For example, cod liver oil, shark liver oils, and whale oil such as spermaceti exemplify several of the fish oils which may be used herein. A number of branched chain oils are synthesized biochemically in 5-carbon isoprene units and are generally referred to as terpenoids. Shark liver oil contains a branched, unsaturated terpenoids known as squalene, 2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene, which is particularly preferred herein. Squalane, the saturated analog to squalene, is also a preferred oil. Fish oils, including squalene and squalane, are readily available from commercial sources or may be obtained by methods known in the art. Other preferred oils are the tocopherols (see below). Mixtures of oils can be used.

Surfactants can be classified by their 'HLB' (hydrophile/lipophile balance). Preferred surfactants of the invention have a HLB of at least 10, preferably at least 15, and more preferably at least 16. The invention can be used with surfactants including, but not limited to: the polyoxyethylene sorbitan esters surfactants (commonly referred to as the Tweens), especially polysorbate 20 and polysorbate 80; copolymers of ethylene oxide (EO), propylene oxide (PO), and/or butylene oxide (BO), sold under the DOWFAX™ tradename, such as linear EO/PO block copolymers; octoxynols, which can vary in the number of repeating ethoxy (oxy-1,2-ethanediyl) groups, with octoxynol-9 (Triton X-100, or t-octylphenoxypolyethoxyethanol) being of particular interest; (octylphenoxy)polyethoxyethanol (IGEPAL CA-630/NP-40); phospholipids such as phosphatidylcholine (lecithin); polyoxyethylene fatty ethers derived from lauryl, cetyl, stearyl and oleyl alcohols (known as Brij surfactants), such as triethyleneglycol monolauryl ether (Brij 30); and sorbitan esters (commonly known as the SPANs), such as sorbitan trioleate (Span 85) and sorbitan monolaurate. Preferred surfactants for including in the emulsion are Tween 80 (polyoxyethylene sorbitan monooleate), Span 85 (sorbitan trioleate), lecithin and Triton X-100. As mentioned above, detergents such as Tween 80 may contribute to the thermal stability seen in the examples below.

Mixtures of surfactants can be used *e.g.* Tween 80/Span 85 mixtures. A combination of a polyoxyethylene sorbitan ester such as polyoxyethylene sorbitan monooleate (Tween 80) and an octoxynol such as t-octylphenoxypolyethoxyethanol (Triton X-100) is also suitable. Another useful combination comprises laureth 9 plus a polyoxyethylene sorbitan ester and/or an octoxynol.

Preferred amounts of surfactants (% by weight) are: polyoxyethylene sorbitan esters (such as Tween 80) 0.01 to 1%, in particular about 0.1 %; octyl- or nonylphenoxy polyoxyethanols (such as Triton

X-100, or other detergents in the Triton series) 0.001 to 0.1 %, in particular 0.005 to 0.02%; polyoxyethylene ethers (such as laureth 9) 0.1 to 20 %, preferably 0.1 to 10 % and in particular 0.1 to 1 % or about 0.5%.

Specific oil-in-water emulsion adjuvants useful with the invention include, but are not limited to:

- 5 • A submicron emulsion of squalene, Tween 80, and Span 85. The composition of the emulsion by volume can be about 5% squalene, about 0.5% polysorbate 80 and about 0.5% Span 85. In weight terms, these ratios become 4.3% squalene, 0.5% polysorbate 80 and 0.48% Span 85. This adjuvant is known as 'MF59' [98-100], as described in more detail in Chapter 10 of ref. 101 and chapter 12 of ref. 102. The MF59 emulsion advantageously includes citrate ions
10 *e.g.* 10mM sodium citrate buffer.
- An emulsion of squalene, a tocopherol, and Tween 80. The emulsion may include phosphate buffered saline. It may also include Span 85 (*e.g.* at 1%) and/or lecithin. These emulsions may have from 2 to 10% squalene, from 2 to 10% tocopherol and from 0.3 to 3% Tween 80, and the weight ratio of squalene:tocopherol is preferably ≤ 1 as this provides a more stable emulsion.
15 Squalene and Tween 80 may be present volume ratio of about 5:2. One such emulsion can be made by dissolving Tween 80 in PBS to give a 2% solution, then mixing 90ml of this solution with a mixture of (5g of DL- α -tocopherol and 5ml squalene), then microfluidising the mixture. The resulting emulsion may have submicron oil droplets *e.g.* with an average diameter of between 100 and 250nm, preferably about 180nm.
- 20 • An emulsion of squalene, a tocopherol, and a Triton detergent (*e.g.* Triton X-100). The emulsion may also include a 3d-MPL (see below). The emulsion may contain a phosphate buffer.
- An emulsion comprising a polysorbate (*e.g.* polysorbate 80), a Triton detergent (*e.g.* Triton X-100) and a tocopherol (*e.g.* an α -tocopherol succinate). The emulsion may include these
25 three components at a mass ratio of about 75:11:10 (*e.g.* 750 μ g/ml polysorbate 80, 110 μ g/ml Triton X-100 and 100 μ g/ml α -tocopherol succinate), and these concentrations should include any contribution of these components from antigens. The emulsion may also include squalene. The emulsion may also include a 3d-MPL (see below). The aqueous phase may contain a phosphate buffer.
- 30 • An emulsion of squalene, polysorbate 80 and poloxamer 401 ("Pluronic™ L121"). The emulsion can be formulated in phosphate buffered saline, pH 7.4. This emulsion is a useful delivery vehicle for muramyl dipeptides, and has been used with threonyl-MDP in the "SAF-1" adjuvant [103] (0.05-1% Thr-MDP, 5% squalene, 2.5% Pluronic L121 and 0.2% polysorbate 80). It can also be used without the Thr-MDP, as in the "AF" adjuvant [104] (5%
35 squalene, 1.25% Pluronic L121 and 0.2% polysorbate 80). Microfluidisation is preferred.
- An emulsion comprising squalene, an aqueous solvent, a polyoxyethylene alkyl ether hydrophilic nonionic surfactant (*e.g.* polyoxyethylene (12) cetostearyl ether) and a

hydrophobic nonionic surfactant (*e.g.* a sorbitan ester or mannide ester, such as sorbitan monooleate or 'Span 80'). The emulsion is preferably thermoreversible and/or has at least 90% of the oil droplets (by volume) with a size less than 200 nm [105]. The emulsion may also include one or more of: alditol; a cryoprotective agent (*e.g.* a sugar, such as dodecylmaltoside and/or sucrose); and/or an alkylpolyglycoside. Such emulsions may be lyophilized.

- An emulsion having from 0.5-50% of an oil, 0.1-10% of a phospholipid, and 0.05-5% of a non-ionic surfactant. As described in reference 106, preferred phospholipid components are phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidylglycerol, phosphatidic acid, sphingomyelin and cardiolipin. Submicron droplet sizes are advantageous.
- A submicron oil-in-water emulsion of a non-metabolisable oil (such as light mineral oil) and at least one surfactant (such as lecithin, Tween 80 or Span 80). Additives may be included, such as QuilA saponin, cholesterol, a saponin-lipophile conjugate (such as GPI-0100, described in reference 107, produced by addition of aliphatic amine to desacylsaponin via the carboxyl group of glucuronic acid), dimethyldioctadecylammonium bromide and/or N,N-dioctadecyl-N,N-bis (2-hydroxyethyl)propanediamine.
- An emulsion comprising a mineral oil, a non-ionic lipophilic ethoxylated fatty alcohol, and a non-ionic hydrophilic surfactant (*e.g.* an ethoxylated fatty alcohol and/or polyoxyethylene-polyoxypropylene block copolymer) [108].
- An emulsion comprising a mineral oil, a non-ionic hydrophilic ethoxylated fatty alcohol, and a non-ionic lipophilic surfactant (*e.g.* an ethoxylated fatty alcohol and/or polyoxyethylene-polyoxypropylene block copolymer) [108].
- An emulsion in which a saponin (*e.g.* QuilA or QS21) and a sterol (*e.g.* a cholesterol) are associated as helical micelles [109].

The emulsions may be mixed with antigen extemporaneously, at the time of delivery. Thus the adjuvant and antigen may be kept separately in a packaged or distributed vaccine, ready for final formulation at the time of use. The antigen will generally be in an aqueous form, such that the vaccine is finally prepared by mixing two liquids. The volume ratio of the two liquids for mixing can vary (*e.g.* between 5:1 and 1:5) but is generally about 1:1.

After the antigen and adjuvant have been mixed, haemagglutinin antigen will generally remain in aqueous solution but may distribute itself around the oil/water interface. In general, little if any haemagglutinin will enter the oil phase of the emulsion.

Where a composition includes a tocopherol, any of the α , β , γ , δ , ϵ or ξ tocopherols can be used, but α -tocopherols are preferred. The tocopherol can take several forms *e.g.* different salts and/or isomers.

Salts include organic salts, such as succinate, acetate, nicotinate, *etc.* D- α -tocopherol and DL- α -tocopherol can both be used. Tocopherols are advantageously included in vaccines for use in

elderly patients (e.g. aged 60 years or older) because vitamin E has been reported to have a positive effect on the immune response in this patient group [110]. They also have antioxidant properties that may help to stabilize the emulsions [111]. A preferred α -tocopherol is DL- α -tocopherol, and the preferred salt of this tocopherol is the succinate. The succinate salt has been found to cooperate with
5 TNF-related ligands *in vivo*. Moreover, α -tocopherol succinate is known to be compatible with influenza vaccines and to be a useful preservative as an alternative to mercurial compounds [8].

Cytokine-inducing agents

Cytokine-inducing agents for inclusion in compositions of the invention are able, when administered to a patient, to elicit the immune system to release cytokines, including interferons and interleukins.

10 Cytokine responses are known to be involved in the early and decisive stages of host defense against influenza infection [112]. Preferred agents can elicit the release of one or more of: interferon- γ ; interleukin-1; interleukin-2; interleukin-12; TNF- α ; TNF- β ; and GM-CSF. Preferred agents elicit the release of cytokines associated with a Th1-type immune response e.g. interferon- γ , TNF- α , interleukin-2. Stimulation of both interferon- γ and interleukin-2 is preferred.

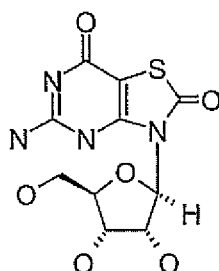
15 As a result of receiving a composition of the invention, therefore, a patient will have T cells that, when stimulated with an influenza antigen, will release the desired cytokine(s) in an antigen-specific manner. For example, T cells purified from their blood will release γ -interferon when exposed *in vitro* to influenza virus haemagglutinin. Methods for measuring such responses in peripheral blood mononuclear cells (PBMC) are known in the art, and include ELISA, ELISPOT, flow-cytometry and
20 real-time PCR. For example, reference 113 reports a study in which antigen-specific T cell-mediated immune responses against tetanus toxoid, specifically γ -interferon responses, were monitored, and found that ELISPOT was the most sensitive method to discriminate antigen-specific TT-induced responses from spontaneous responses, but that intracytoplasmic cytokine detection by flow cytometry was the most efficient method to detect re-stimulating effects.

25 Suitable cytokine-inducing agents include, but are not limited to:

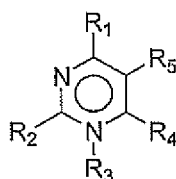
- An immunostimulatory oligonucleotide, such as one containing a CpG motif (a dinucleotide sequence containing an unmethylated cytosine linked by a phosphate bond to a guanosine), or a double-stranded RNA, or an oligonucleotide containing a palindromic sequence, or an oligonucleotide containing a poly(dG) sequence.
- 30 • 3-O-deacylated monophosphoryl lipid A ('3dMPL', also known as 'MPLTM') [114-117].
- An imidazoquinoline compound, such as Imiquimod ("R-837") [118,119], Resiquimod ("R-848") [120], and their analogs; and salts thereof (e.g. the hydrochloride salts). Further details about immunostimulatory imidazoquinolines can be found in references 121 to 125.
- A thiosemicarbazone compound, such as those disclosed in reference 126. Methods of
35 formulating, manufacturing, and screening for active compounds are also described in

reference 126. The thiosemicarbazones are particularly effective in the stimulation of human peripheral blood mononuclear cells for the production of cytokines, such as TNF- α .

- A tryptanthrin compound, such as those disclosed in reference 127. Methods of formulating, manufacturing, and screening for active compounds are also described in reference 127.
- A nucleoside analog, such as: (a) Isatorabine (ANA-245; 7-thia-8-oxoguanosine):



and prodrugs thereof; (b) ANA975; (c) ANA-025-1; (d) ANA380; (e) the compounds disclosed in references 128 to 130; (f) a compound having the formula:

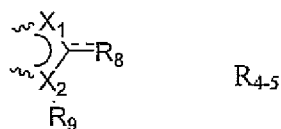


wherein:

R₁ and R₂ are each independently H, halo, -NR_aR_b, -OH, C₁₋₆ alkoxy, substituted C₁₋₆ alkoxy, heterocyclyl, substituted heterocyclyl, C₆₋₁₀ aryl, substituted C₆₋₁₀ aryl, C₁₋₆ alkyl, or substituted C₁₋₆ alkyl;

R₃ is absent, H, C₁₋₆ alkyl, substituted C₁₋₆ alkyl, C₆₋₁₀ aryl, substituted C₆₋₁₀ aryl, heterocyclyl, or substituted heterocyclyl;

R₄ and R₅ are each independently H, halo, heterocyclyl, substituted heterocyclyl, -C(O)-R_d, C₁₋₆ alkyl, substituted C₁₋₆ alkyl, or bound together to form a 5 membered ring as in R₄₋₅:

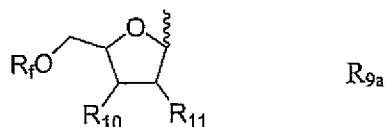


the binding being achieved at the bonds indicated by a ~~~

X₁ and X₂ are each independently N, C, O, or S;

R₈ is H, halo, -OH, C₁₋₆ alkyl, C₂₋₆ alkenyl, C₂₋₆ alkynyl, -OH, -NR_aR_b, -(CH₂)_n-O-R_c, -O-(C₁₋₆ alkyl), -S(O)_pR_e, or -C(O)-R_d;

R₉ is H, C₁₋₆ alkyl, substituted C₁₋₆ alkyl, heterocyclyl, substituted heterocyclyl or R_{9a},
wherein R_{9a} is:



the binding being achieved at the bond indicated by a ~~~

- 5 R₁₀ and R₁₁ are each independently H, halo, C₁₋₆ alkoxy, substituted C₁₋₆ alkoxy, -NR_aR_b, or -OH;
each R_a and R_b is independently H, C₁₋₆ alkyl, substituted C₁₋₆ alkyl, -C(O)R_d, C₆₋₁₀ aryl;
each R_c is independently H, phosphate, diphosphate, triphosphate, C₁₋₆ alkyl, or substituted C₁₋₆ alkyl;
- 10 each R_d is independently H, halo, C₁₋₆ alkyl, substituted C₁₋₆ alkyl, C₁₋₆ alkoxy, substituted C₁₋₆ alkoxy, -NH₂, -NH(C₁₋₆ alkyl), -NH(substituted C₁₋₆ alkyl), -N(C₁₋₆ alkyl)₂, -N(substituted C₁₋₆ alkyl)₂, C₆₋₁₀ aryl, or heterocyclyl;
each R_e is independently H, C₁₋₆ alkyl, substituted C₁₋₆ alkyl, C₆₋₁₀ aryl, substituted C₆₋₁₀ aryl, heterocyclyl, or substituted heterocyclyl;
- 15 each R_f is independently H, C₁₋₆ alkyl, substituted C₁₋₆ alkyl, -C(O)R_d, phosphate, diphosphate, or triphosphate;
each n is independently 0, 1, 2, or 3;
each p is independently 0, 1, or 2; or

or (g) a pharmaceutically acceptable salt of any of (a) to (f), a tautomer of any of (a) to (f), or
20 a pharmaceutically acceptable salt of the tautomer.

- Loxoribine (7-allyl-8-oxoguanosine) [131].
- Compounds disclosed in reference 132, including: Acylpiperazine compounds, Indoleione compounds, Tetrahydroisoquinoline (THIQ) compounds, Benzocyclodione compounds, Aminoazavinyl compounds, Aminobenzimidazole quinolinone (ABIQ) compounds
25 [133,134], Hydraphtalamide compounds, Benzophenone compounds, Isoxazole compounds, Sterol compounds, Quinazolinone compounds, Pyrrole compounds [135], Anthraquinone compounds, Quinoxaline compounds, Triazine compounds, Pyrazalopyrimidine compounds, and Benzazole compounds [136].
- A polyoxidonium polymer [137,138] or other N-oxidized polyethylene-piperazine derivative.
- 30 • Compounds disclosed in reference 139.
- An aminoalkyl glucosaminide phosphate derivative, such as RC-529 [140,141].
- A CD1d ligand, such as an α -glycosylceramide [142-149] (e.g. α -galactosylceramide), phytosphingosine-containing α -glycosylceramides, OCH, KRN7000 [(2S,3S,4R)-1-O-(α -D-

galactopyranosyl)-2-(N-hexacosanoylamino)-1,3,4-octadecanetriol], CRONY-101, 3''-O-sulfo-galactosylceramide, *etc.*

- A phosphazene, such as poly[di(carboxylatophenoxy)phosphazene] ("PCPP") as described, for example, in references 150 and 151.

- 5 • Small molecule immunopotentiators (SMIPs) such as:

N2-methyl-1-(2-methylpropyl)-1H-imidazo[4,5-c]quinoline-2,4-diamine

N2,N2-dimethyl-1-(2-methylpropyl)-1H-imidazo[4,5-c]quinoline-2,4-diamine

N2-ethyl-N2-methyl-1-(2-methylpropyl)-1H-imidazo[4,5-c]quinoline-2,4-diamine

N2-methyl-1-(2-methylpropyl)-N2-propyl-1H-imidazo[4,5-c]quinoline-2,4-diamine

10 1-(2-methylpropyl)-N2-propyl-1H-imidazo[4,5-c]quinoline-2,4-diamine

N2-butyl-1-(2-methylpropyl)-1H-imidazo[4,5-c]quinoline-2,4-diamine

N2-butyl-N2-methyl-1-(2-methylpropyl)-1H-imidazo[4,5-c]quinoline-2,4-diamine

N2-methyl-1-(2-methylpropyl)-N2-pentyl-1H-imidazo[4,5-c]quinoline-2,4-diamine

N2-methyl-1-(2-methylpropyl)-N2-prop-2-enyl-1H-imidazo[4,5-c]quinoline-2,4-diamine

15 1-(2-methylpropyl)-2-[(phenylmethyl)thio]-1H-imidazo[4,5-c]quinolin-4-amine

1-(2-methylpropyl)-2-(propylthio)-1H-imidazo[4,5-c]quinolin-4-amine

2-[[4-amino-1-(2-methylpropyl)-1H-imidazo[4,5-c]quinolin-2-yl](methylamino)ethanol

2-[[4-amino-1-(2-methylpropyl)-1H-imidazo[4,5-c]quinolin-2-yl](methylamino)ethyl acetate

4-amino-1-(2-methylpropyl)-1,3-dihydro-2H-imidazo[4,5-c]quinolin-2-one

20 N2-butyl-1-(2-methylpropyl)-N4,N4-bis(phenylmethyl)-1H-imidazo[4,5-c]quinoline-2,4-diamine

N2-butyl-N2-methyl-1-(2-methylpropyl)-N4,N4-bis(phenylmethyl)-1H-imidazo[4,5-c]quinoline-2,4-diamine

N2-methyl-1-(2-methylpropyl)-N4,N4-bis(phenylmethyl)-1H-imidazo[4,5-c]quinoline-2,4-diamine

25 N2,N2-dimethyl-1-(2-methylpropyl)-N4,N4-bis(phenylmethyl)-1H-imidazo[4,5-c]quinoline-2,4-diamine

1-{4-amino-2-[methyl(propyl)amino]-1H-imidazo[4,5-c]quinolin-1-yl}-2-methylpropan-2-ol

1-[4-amino-2-(propylamino)-1H-imidazo[4,5-c]quinolin-1-yl]-2-methylpropan-2-ol

30 N4,N4-dibenzyl-1-(2-methoxy-2-methylpropyl)-N2-propyl-1H-imidazo[4,5-c]quinoline-2,4-diamine.

The cytokine-inducing agents for use in the present invention may be modulators and/or agonists of Toll-Like Receptors (TLR). For example, they may be agonists of one or more of the human TLR1, TLR2, TLR3, TLR4, TLR7, TLR8, and/or TLR9 proteins. Preferred agents are agonists of TLR7 (*e.g.* imidazoquinolines) and/or TLR9 (*e.g.* CpG oligonucleotides). These agents are useful for activating innate immunity pathways.

The cytokine-inducing agent can be added to the composition at various stages during its production. For example, it may be within an antigen composition, and this mixture can then be added to an oil-in-water emulsion. As an alternative, it may be within an oil-in-water emulsion, in which case the agent can either be added to the emulsion components before emulsification, or it can be added to the emulsion after emulsification. Similarly, the agent may be coacervated within the emulsion droplets. The location and distribution of the cytokine-inducing agent within the final composition will depend on its hydrophilic/lipophilic properties *e.g.* the agent can be located in the aqueous phase, in the oil phase, and/or at the oil-water interface.

The cytokine-inducing agent can be conjugated to a separate agent, such as an antigen (*e.g.* CRM197). A general review of conjugation techniques for small molecules is provided in ref. 152. As an alternative, the adjuvants may be non-covalently associated with additional agents, such as by way of hydrophobic or ionic interactions.

Two preferred cytokine-inducing agents are (a) immunostimulatory oligonucleotides and (b) 3dMPL. Immunostimulatory oligonucleotides can include nucleotide modifications/analogues such as phosphorothioate modifications and can be double-stranded or (except for RNA) single-stranded. References 153, 154 and 155 disclose possible analog substitutions *e.g.* replacement of guanosine with 2'-deoxy-7-deazaguanosine. The adjuvant effect of CpG oligonucleotides is further discussed in refs. 156-161. A CpG sequence may be directed to TLR9, such as the motif GTCGTT or TTCGTT [162]. The CpG sequence may be specific for inducing a Th1 immune response, such as a CpG-A ODN (oligodeoxynucleotide), or it may be more specific for inducing a B cell response, such as a CpG-B ODN. CpG-A and CpG-B ODNs are discussed in refs. 163-165. Preferably, the CpG is a CpG-A ODN. Preferably, the CpG oligonucleotide is constructed so that the 5' end is accessible for receptor recognition. Optionally, two CpG oligonucleotide sequences may be attached at their 3' ends to form "immunomers". See, for example, references 162 & 166-168. A useful CpG adjuvant is CpG7909, also known as ProMune™ (Coley Pharmaceutical Group, Inc.).

As an alternative, or in addition, to using CpG sequences, TpG sequences can be used [169]. These oligonucleotides may be free from unmethylated CpG motifs.

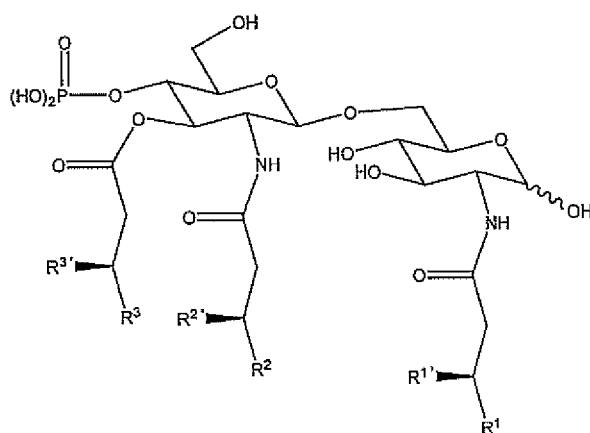
The immunostimulatory oligonucleotide may be pyrimidine-rich. For example, it may comprise more than one consecutive thymidine nucleotide (*e.g.* TTTT, as disclosed in ref. 169), and/or it may have a nucleotide composition with >25% thymidine (*e.g.* >35%, >40%, >50%, >60%, >80%, *etc.*). For example, it may comprise more than one consecutive cytosine nucleotide (*e.g.* CCCC, as disclosed in ref. 169), and/or it may have a nucleotide composition with >25% cytosine (*e.g.* >35%, >40%, >50%, >60%, >80%, *etc.*). These oligonucleotides may be free from unmethylated CpG motifs.

Immunostimulatory oligonucleotides will typically comprise at least 20 nucleotides. They may comprise fewer than 100 nucleotides.

3dMPL (also known as 3 de-O-acylated monophosphoryl lipid A or 3-O-desacyl-4'-monophosphoryl lipid A) is an adjuvant in which position 3 of the reducing end glucosamine in monophosphoryl lipid

A has been de-acylated. 3dMPL has been prepared from a heptoseless mutant of *Salmonella minnesota*, and is chemically similar to lipid A but lacks an acid-labile phosphoryl group and a base-labile acyl group. It activates cells of the monocyte/macrophage lineage and stimulates release of several cytokines, including IL-1, IL-12, TNF- α and GM-CSF (see also ref. 170). Preparation of
5 3dMPL was originally described in reference 171.

3dMPL can take the form of a mixture of related molecules, varying by their acylation (*e.g.* having 3, 4, 5 or 6 acyl chains, which may be of different lengths). The two glucosamine (also known as 2-deoxy-2-amino-glucose) monosaccharides are N-acylated at their 2-position carbons (*i.e.* at positions 2 and 2'), and there is also O-acylation at the 3' position. The group attached to carbon 2 has formula -NH-CO-CH₂-CR¹R¹. The group attached to carbon 2' has formula -NH-CO-CH₂-CR²R².
10 The group attached to carbon 3' has formula -O-CO-CH₂-CR³R³. A representative structure is:

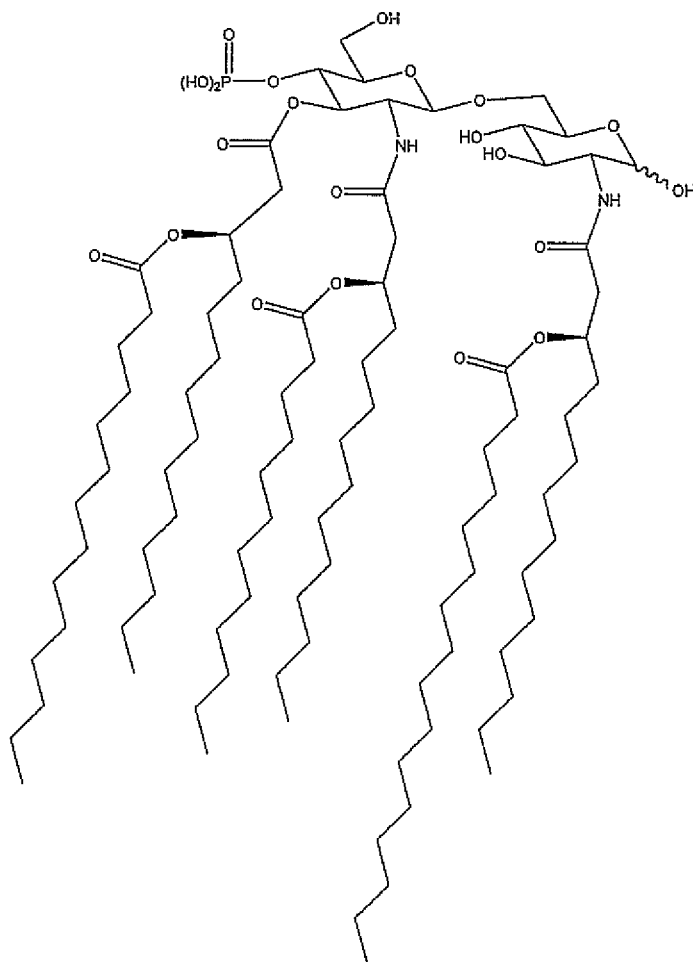


Groups R^1 , R^2 and R^3 are each independently $-(CH_2)_n-CH_3$. The value of n is preferably between 8 and 16, more preferably between 9 and 12, and is most preferably 10.

15 Groups R^1 , R^2 and R^3 can each independently be: (a) -H; (b) -OH; or (c) $-O-CO-R^4$, where R^4 is either -H or $-(CH_2)_m-CH_3$, wherein the value of m is preferably between 8 and 16, and is more preferably 10, 12 or 14. At the 2 position, m is preferably 14. At the 2' position, m is preferably 10. At the 3' position, m is preferably 12. Groups R^1 , R^2 and R^3 are thus preferably -O-acyl groups from dodecanoic acid, tetradecanoic acid or hexadecanoic acid.

20 When all of R^1 , R^2 and R^3 are -H then the 3dMPL has only 3 acyl chains (one on each of positions 2, 2' and 3'). When only two of R^1 , R^2 and R^3 are -H then the 3dMPL can have 4 acyl chains. When only one of R^1 , R^2 and R^3 is -H then the 3dMPL can have 5 acyl chains. When none of R^1 , R^2 and R^3 is -H then the 3dMPL can have 6 acyl chains. The 3dMPL adjuvant used according to the invention can be a mixture of these forms, with from 3 to 6 acyl chains, but it is preferred to include
25 3dMPL with 6 acyl chains in the mixture, and in particular to ensure that the hexaacyl chain form makes up at least 10% by weight of the total 3dMPL *e.g.* $\geq 20\%$, $\geq 30\%$, $\geq 40\%$, $\geq 50\%$ or more. 3dMPL with 6 acyl chains has been found to be the most adjuvant-active form.

Thus the most preferred form of 3dMPL for inclusion in compositions of the invention is:



Where 3dMPL is used in the form of a mixture then references to amounts or concentrations of 3dMPL in compositions of the invention refer to the combined 3dMPL species in the mixture.

In aqueous conditions, 3dMPL can form micellar aggregates or particles with different sizes *e.g.* with a diameter <150nm or >500nm. Either or both of these can be used with the invention, and the better particles can be selected by routine assay. Smaller particles (*e.g.* small enough to give a clear aqueous suspension of 3dMPL) are preferred for use according to the invention because of their superior activity [172]. Preferred particles have a mean diameter less than 220nm, more preferably less than 200nm or less than 150nm or less than 120nm, and can even have a mean diameter less than 100nm. In most cases, however, the mean diameter will not be lower than 50nm. These particles are small enough to be suitable for filter sterilization. Particle diameter can be assessed by the routine technique of dynamic light scattering, which reveals a mean particle diameter. Where a particle is said to have a diameter of x nm, there will generally be a distribution of particles about this mean, but at least 50% by number (*e.g.* $\geq 60\%$, $\geq 70\%$, $\geq 80\%$, $\geq 90\%$, or more) of the particles will have a diameter within the range $x \pm 25\%$.

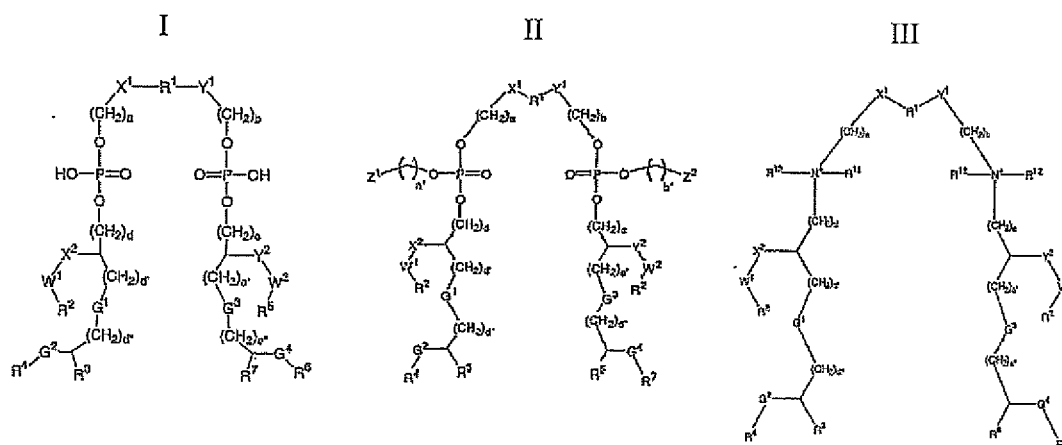
3dMPL can advantageously be used in combination with an oil-in-water emulsion. Substantially all of the 3dMPL may be located in the aqueous phase of the emulsion.

The 3dMPL can be used on its own, or in combination with one or more further compounds. For example, it is known to use 3dMPL in combination with the QS21 saponin [173] (including in an oil-in-water emulsion [174]), with an immunostimulatory oligonucleotide, with both QS21 and an immunostimulatory oligonucleotide, with aluminum phosphate [175], with aluminum hydroxide [176], or with both aluminum phosphate and aluminum hydroxide.

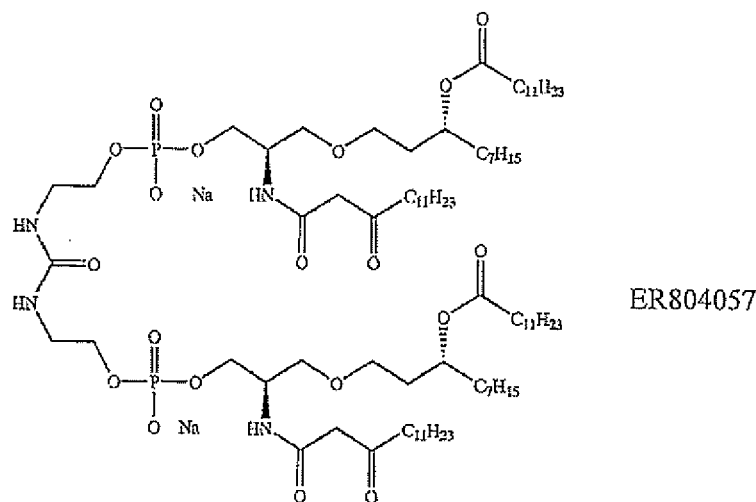
Fatty adjuvants

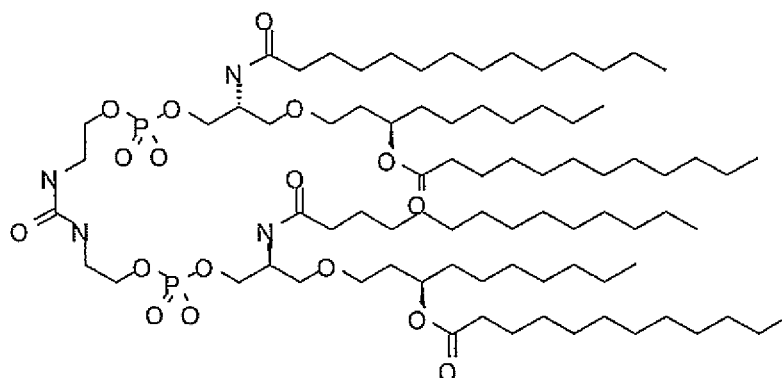
Fatty adjuvants that can be used with the invention include the oil-in-water emulsions described above, and also include, for example:

- A compound of formula I, II or III, or a salt thereof:



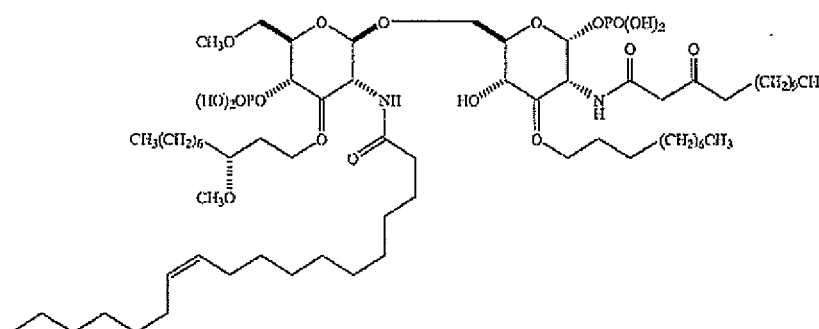
10 as defined in reference 177, such as 'ER 803058', 'ER 803732', 'ER 804053', ER 804058', 'ER 804059', 'ER 804442', 'ER 804680', 'ER 804764', ER 803022 or 'ER 804057' e.g.:





ER-803022:

- Derivatives of lipid A from *Escherichia coli* such as OM-174 (described in refs. 178 & 179).
- A formulation of a cationic lipid and a (usually neutral) co-lipid, such as aminopropyl-dimethyl-myristolexyloxy-propanaminium bromide-diphytanoylphosphatidyl-ethanolamine (“Vaxfectin™”) or aminopropyl-dimethyl-bis-dodecyloxy-propanaminium bromide-dioleoylphosphatidyl-ethanolamine (“GAP-DLRIE:DOPE”). Formulations containing (±)-N-(3-aminopropyl)-N,N-dimethyl-2,3-bis(syn-9-tetradeceneyloxy)-1-propanaminium salts are preferred [180].
- 3-O-deacylated monophosphoryl lipid A (see above).
- Compounds containing lipids linked to a phosphate-containing acyclic backbone, such as the TLR4 antagonist E5564 [181,182]:



Aluminum salt adjuvants

The adjuvants known as aluminum hydroxide and aluminum phosphate may be used. These names are conventional, but are used for convenience only, as neither is a precise description of the actual chemical compound which is present (*e.g.* see chapter 9 of reference 101). The invention can use any of the “hydroxide” or “phosphate” adjuvants that are in general use as adjuvants.

The adjuvants known as “aluminium hydroxide” are typically aluminium oxyhydroxide salts, which are usually at least partially crystalline. Aluminium oxyhydroxide, which can be represented by the formula AlO(OH), can be distinguished from other aluminium compounds, such as aluminium hydroxide Al(OH)₃, by infrared (IR) spectroscopy, in particular by the presence of an adsorption band at 1070cm⁻¹ and a strong shoulder at 3090–3100cm⁻¹ [chapter 9 of ref. 101]. The degree of crystallinity of an aluminium hydroxide adjuvant is reflected by the width of the diffraction band at

half height (WHH), with poorly-crystalline particles showing greater line broadening due to smaller crystallite sizes. The surface area increases as WHH increases, and adjuvants with higher WHH values have been seen to have greater capacity for antigen adsorption. A fibrous morphology (*e.g.* as seen in transmission electron micrographs) is typical for aluminium hydroxide adjuvants. The pI of aluminium hydroxide adjuvants is typically about 11 *i.e.* the adjuvant itself has a positive surface charge at physiological pH. Adsorptive capacities of between 1.8-2.6 mg protein per mg Al⁺⁺⁺ at pH 7.4 have been reported for aluminium hydroxide adjuvants.

The adjuvants known as "aluminium phosphate" are typically aluminium hydroxyphosphates, often also containing a small amount of sulfate (*i.e.* aluminium hydroxyphosphate sulfate). They may be obtained by precipitation, and the reaction conditions and concentrations during precipitation influence the degree of substitution of phosphate for hydroxyl in the salt. Hydroxyphosphates generally have a PO₄/Al molar ratio between 0.3 and 1.2. Hydroxyphosphates can be distinguished from strict AlPO₄ by the presence of hydroxyl groups. For example, an IR spectrum band at 3164cm⁻¹ (*e.g.* when heated to 200°C) indicates the presence of structural hydroxyls [ch.9 of ref. 101]

The PO₄/Al³⁺ molar ratio of an aluminium phosphate adjuvant will generally be between 0.3 and 1.2, preferably between 0.8 and 1.2, and more preferably 0.95±0.1. The aluminium phosphate will generally be amorphous, particularly for hydroxyphosphate salts. A typical adjuvant is amorphous aluminium hydroxyphosphate with PO₄/Al molar ratio between 0.84 and 0.92, included at 0.6mg Al³⁺/ml. The aluminium phosphate will generally be particulate (*e.g.* plate-like morphology as seen in transmission electron micrographs). Typical diameters of the particles are in the range 0.5-20µm (*e.g.* about 5-10µm) after any antigen adsorption. Adsorptive capacities of between 0.7-1.5 mg protein per mg Al⁺⁺⁺ at pH 7.4 have been reported for aluminium phosphate adjuvants.

The point of zero charge (PZC) of aluminium phosphate is inversely related to the degree of substitution of phosphate for hydroxyl, and this degree of substitution can vary depending on reaction conditions and concentration of reactants used for preparing the salt by precipitation. PZC is also altered by changing the concentration of free phosphate ions in solution (more phosphate = more acidic PZC) or by adding a buffer such as a histidine buffer (makes PZC more basic). Aluminium phosphates used according to the invention will generally have a PZC of between 4.0 and 7.0, more preferably between 5.0 and 6.5 *e.g.* about 5.7.

Suspensions of aluminium salts used to prepare compositions of the invention may contain a buffer (*e.g.* a phosphate or a histidine or a Tris buffer), but this is not always necessary. The suspensions are preferably sterile and pyrogen-free. A suspension may include free aqueous phosphate ions *e.g.* present at a concentration between 1.0 and 20 mM, preferably between 5 and 15 mM, and more preferably about 10 mM. The suspensions may also comprise sodium chloride.

The invention can use a mixture of both an aluminium hydroxide and an aluminium phosphate [62]. In this case there may be more aluminium phosphate than hydroxide *e.g.* a weight ratio of at least 2:1 *e.g.* ≥5:1, ≥6:1, ≥7:1, ≥8:1, ≥9:1, *etc.*

The concentration of Al⁺⁺⁺ in a composition for administration to a patient is preferably less than 10mg/ml *e.g.* ≤ 5 mg/ml, ≤ 4 mg/ml, ≤ 3 mg/ml, ≤ 2 mg/ml, ≤ 1 mg/ml, *etc.* A preferred range is between 0.3 and 1mg/ml. A maximum of 0.85mg/dose is preferred.

As well as including one or more aluminium salt adjuvants, the adjuvant component may include one or more further adjuvant or immunostimulating agents. Such additional components include, but are not limited to: a 3-O-deacylated monophosphoryl lipid A adjuvant ('3d-MPL'); and/or an oil-in-water emulsion. 3d-MPL has also been referred to as 3 de-O-acylated monophosphoryl lipid A or as 3-O-desacyl-4'-monophosphoryl lipid A. The name indicates that position 3 of the reducing end glucosamine in monophosphoryl lipid A is de-acylated. It has been prepared from a heptoseless mutant of *S.minnesota*, and is chemically similar to lipid A but lacks an acid-labile phosphoryl group and a base-labile acyl group. It activates cells of the monocyte/macrophage lineage and stimulates release of several cytokines, including IL-1, IL-12, TNF- α and GM-CSF. Preparation of 3d-MPL was originally described in reference 171, and the product has been manufactured and sold by Corixa Corporation under the name MPLTM. Further details can be found in refs 114 to 117.

15 *Pharmaceutical compositions*

Compositions of the invention are pharmaceutically acceptable. They usually include components in addition to the antigens *e.g.* they typically include one or more pharmaceutical carrier(s) and/or excipient(s). A thorough discussion of such components is available in reference 183.

Compositions will generally be in aqueous form.

20 The composition includes no mercurial material. It may include a preservative such as 2-phenoxyethanol, but preservative-free vaccines are more preferred.

To control tonicity, it is preferred to include a physiological salt, such as a sodium salt. Sodium chloride (NaCl) is preferred, which may be present at between 1 and 20 mg/ml. Other salts that may be present include potassium chloride, potassium dihydrogen phosphate, disodium phosphate dehydrate, magnesium chloride, calcium chloride, *etc.*

Compositions will generally have an osmolality of between 200 mOsm/kg and 400 mOsm/kg, preferably between 240-360 mOsm/kg, and will more preferably fall within the range of 290-310 mOsm/kg. Osmolality has previously been reported not to have an impact on pain caused by vaccination [184], but keeping osmolality in this range is nevertheless preferred.

30 Compositions may include one or more buffers. Typical buffers include: a phosphate buffer; a Tris buffer; a borate buffer; a succinate buffer; a histidine buffer; or a citrate buffer. Buffers will typically be included in the 5-20mM range. The buffer may be in the emulsion's aqueous phase.

The pH of a composition will generally be between 5.0 and 8.1, and more typically between 6.0 and 8.0 *e.g.* 6.5 and 7.5, or between 7.0 and 7.8. A process of the invention may therefore include a step of adjusting the pH of the bulk vaccine prior to packaging.

The composition is preferably sterile. The composition is preferably gluten free.

The vaccine is free from antibiotics (*e.g.* neomycin, kanamycin, polymyxin B).

5 The composition may include material for a single immunisation, or may include material for multiple immunisations (*i.e.* a 'multidose' composition). Multidose arrangements usually include a preservative in the vaccine. To avoid this need, a vaccine may be contained in a container having an aseptic adaptor for removal of material.

Influenza vaccines are typically administered in a dosage volume of about 0.5ml, although a half dose (*i.e.* about 0.25ml) may be administered to children, and unit doses will be selected accordingly *e.g.* a unit dose to give a 0.5ml dose for administration to a patient.

10 *Packaging of compositions or kit components*

Processes of the invention can include a step in which vaccine is placed into a container, and in particular into a container for distribution for use by physicians. After packaging into such containers, the container is not refrigerated.

15 Suitable containers for the vaccines include vials, nasal sprays and disposable syringes, which should be sterile.

Where a composition/component is located in a vial, the vial is preferably made of a glass or plastic material. The vial is preferably sterilized before the composition is added to it. To avoid problems with latex-sensitive patients, vials are preferably sealed with a latex-free stopper, and the absence of latex in all packaging material is preferred. The vial may include a single dose of vaccine, or it may
20 include more than one dose (a 'multidose' vial) *e.g.* 10 doses. Preferred vials are made of colorless glass.

A vial can have a cap (*e.g.* a Luer lock) adapted such that a pre-filled syringe can be inserted into the cap, the contents of the syringe can be expelled into the vial, and the contents of the vial can be removed back into the syringe. After removal of the syringe from the vial, a needle can then be
25 attached and the composition can be administered to a patient. The cap is preferably located inside a seal or cover, such that the seal or cover has to be removed before the cap can be accessed. A vial may have a cap that permits aseptic removal of its contents, particularly for multidose vials.

Where a composition/component is packaged into a syringe, the syringe may have a needle attached to it. If a needle is not attached, a separate needle may be supplied with the syringe for assembly and
30 use. Such a needle may be sheathed. Safety needles are preferred. 1-inch 23-gauge, 1-inch 25-gauge and 5/8-inch 25-gauge needles are typical. Syringes may be provided with peel-off labels on which the lot number, influenza season and expiration date of the contents may be printed, to facilitate record keeping. The plunger in the syringe preferably has a stopper to prevent the plunger from being accidentally removed during aspiration. The syringes may have a latex rubber cap and/or plunger.
35 Disposable syringes contain a single dose of vaccine. The syringe will generally have a tip cap to seal the tip prior to attachment of a needle, and the tip cap is preferably made of a butyl rubber. If the

syringe and needle are packaged separately then the needle is preferably fitted with a butyl rubber shield. Preferred syringes are those marketed under the trade name "Tip-Lok"TM.

Containers may be marked to show a half-dose volume *e.g.* to facilitate delivery to children. For instance, a syringe containing a 0.5ml dose may have a mark showing a 0.25ml volume.

- 5 Where a glass container (*e.g.* a syringe or a vial) is used, then it is preferred to use a container made from a borosilicate glass rather than from a soda lime glass.

A composition may be combined (*e.g.* in the same box) with a leaflet including details of the vaccine *e.g.* instructions for administration, details of the antigens within the vaccine, *etc.* The instructions may also contain warnings *e.g.* to keep a solution of adrenaline readily available in case of
10 anaphylactic reaction following vaccination, *etc.*

Methods of treatment, and administration of the vaccine

Compositions of the invention are suitable for administration to human patients, and the invention provides a method of raising an immune response in a patient, comprising the step of administering a composition of the invention to the patient.

- 15 The invention also provides a kit or composition of the invention for use as a medicament.

The immune response raised by the methods and uses of the invention will generally include an antibody response, preferably a protective antibody response. Methods for assessing antibody responses, neutralising capability and protection after influenza virus vaccination are well known in the art. Human studies have shown that antibody titers against hemagglutinin of human influenza
20 virus are correlated with protection (a serum sample hemagglutination-inhibition titer of about 30–40 gives around 50% protection from infection by a homologous virus) [185]. Antibody responses are typically measured by hemagglutination inhibition, by microneutralisation, by single radial immunodiffusion (SRID), and/or by single radial hemolysis (SRH). These assay techniques are well known in the art.

- 25 Compositions of the invention can be administered in various ways. The most preferred immunisation route is by intramuscular injection (*e.g.* into the arm or leg), but other available routes include subcutaneous injection, intranasal [186-188], oral [189], intradermal [190,191], transcutaneous, transdermal [192], *etc.*

Vaccines prepared according to the invention may be used to treat both children and adults. Influenza
30 vaccines are currently recommended for use in pediatric and adult immunisation, from the age of 6 months. Thus the patient may be less than 1 year old, 1-5 years old, 5-15 years old, 15-55 years old, or at least 55 years old. Preferred patients for receiving the vaccines are the elderly (*e.g.* ≥ 50 years old, ≥ 60 years old, and preferably ≥ 65 years), the young (*e.g.* ≤ 5 years old), hospitalised patients, healthcare workers, armed service and military personnel, pregnant women, the chronically ill,
35 immunodeficient patients, patients who have taken an antiviral compound (*e.g.* an oseltamivir or zanamivir compound; see below) in the 7 days prior to receiving the vaccine, people with egg

allergies and people travelling abroad. The vaccines are not suitable solely for these groups, however, and may be used more generally in a population. For pandemic strains, administration to all age groups is preferred.

5 Preferred compositions of the invention satisfy 1, 2 or 3 of the CPMP criteria for efficacy. In adults (18-60 years), these criteria are: (1) $\geq 70\%$ seroprotection; (2) $\geq 40\%$ seroconversion; and/or (3) a GMT increase of ≥ 2.5 -fold. In elderly (>60 years), these criteria are: (1) $\geq 60\%$ seroprotection; (2) $\geq 30\%$ seroconversion; and/or (3) a GMT increase of ≥ 2 -fold. These criteria are based on open label studies with at least 50 patients.

10 Treatment can be by a single dose schedule or a multiple dose schedule. Multiple doses may be used in a primary immunisation schedule and/or in a booster immunisation schedule. In a multiple dose schedule the various doses may be given by the same or different routes *e.g.* a parenteral prime and mucosal boost, a mucosal prime and parenteral boost, *etc.* Administration of more than one dose (typically two doses) is particularly useful in immunologically naïve patients *e.g.* for people who have never received an influenza vaccine before, or for vaccinating against a new HA subtype (as in
15 a pandemic outbreak). Multiple doses will typically be administered at least 1 week apart (*e.g.* about 2 weeks, about 3 weeks, about 4 weeks, about 6 weeks, about 8 weeks, about 12 weeks, about 16 weeks, *etc.*).

Vaccines produced by the invention may be administered to patients at substantially the same time as (*e.g.* during the same medical consultation or visit to a healthcare professional or vaccination centre)
20 other vaccines *e.g.* at substantially the same time as a measles vaccine, a mumps vaccine, a rubella vaccine, a MMR vaccine, a varicella vaccine, a MMRV vaccine, a diphtheria vaccine, a tetanus vaccine, a pertussis vaccine, a DTP vaccine, a conjugated *H.influenzae* type b vaccine, an inactivated poliovirus vaccine, a hepatitis B virus vaccine, a meningococcal conjugate vaccine (such as a tetravalent A C W135 Y vaccine), a respiratory syncytial virus vaccine, a pneumococcal conjugate
25 vaccine, *etc.* Administration at substantially the same time as a pneumococcal vaccine and/or a meningococcal vaccine is particularly useful in elderly patients.

Similarly, vaccines of the invention may be administered to patients at substantially the same time as (*e.g.* during the same medical consultation or visit to a healthcare professional) an antiviral compound, and in particular an antiviral compound active against influenza virus (*e.g.* oseltamivir
30 and/or zanamivir). These antivirals include neuraminidase inhibitors, such as a (3R,4R,5S)-4-acetylamino-5-amino-3(1-ethylpropoxy)-1-cyclohexene-1-carboxylic acid or 5-(acetylamino)-4-[(aminoiminomethyl)-amino]-2,6-anhydro-3,4,5-trideoxy-D-glycero-D-galactonon-2-enonic acid, including esters thereof (*e.g.* the ethyl esters) and salts thereof (*e.g.* the phosphate salts). A preferred antiviral is (3R,4R,5S)-4-acetylamino-5-amino-3(1-ethylpropoxy)-1-cyclohexene-1-carboxylic acid,
35 ethyl ester, phosphate (1:1), also known as oseltamivir phosphate (TAMIFLU™).

General

The term "comprising" encompasses "including" as well as "consisting" *e.g.* a composition "comprising" X may consist exclusively of X or may include something additional *e.g.* X + Y.

5 The word "substantially" does not exclude "completely" *e.g.* a composition which is "substantially free" from Y may be completely free from Y. Where necessary, the word "substantially" may be omitted from the definition of the invention.

The term "about" in relation to a numerical value x means, for example, $x \pm 10\%$.

10 Unless specifically stated, a process comprising a step of mixing two or more components does not require any specific order of mixing. Thus components can be mixed in any order. Where there are three components then two components can be combined with each other, and then the combination may be combined with the third component, *etc.*

15 Where animal (and particularly bovine) materials are used in the culture of cells, they should be obtained from sources that are free from transmissible spongiform encephalopathies (TSEs), and in particular free from bovine spongiform encephalopathy (BSE). Overall, it is preferred to culture cells in the total absence of animal-derived materials.

Where a compound is administered to the body as part of a composition then that compound may alternatively be replaced by a suitable prodrug.

Where a cell substrate is used for reassortment or reverse genetics procedures, it is preferably one that has been approved for use in human vaccine production *e.g.* as in Ph Eur general chapter 5.2.3.

20 Identity between polypeptide sequences is preferably determined by the Smith-Waterman homology search algorithm as implemented in the MPSRCH program (Oxford Molecular), using an affine gap search with parameters *gap open penalty=12* and *gap extension penalty=1*.

MODES FOR CARRYING OUT THE INVENTION

25 Rather than using eggs, influenza A and B viruses were grown in MDCK cells in a suspension culture, following the teaching of references 27 and 47. The culture did not include any antibiotic.

The final culture medium was clarified to provide virions, which were then subjected to chromatography and ultrafiltration/diafiltration.

30 Rather than use formaldehyde for inactivation, virions in the resulting material were inactivated using β -propiolactone (final concentration 0.05% v/v; incubated for 16-20 hours at 2-8°C, and then hydrolyzed by incubating at 37°C for 2-2.5 hours), following the teaching of reference 59. CTAB was then used to split the virions, and various further processing steps gave a final monovalent bulk vaccine containing purified surface proteins.

The final bulk material contained no mercurial preservative, no an antibiotic, no formaldehyde, and no egg-derived materials.

Individual doses of vaccine were prepared from the bulk, each containing 15µg of HA from a A/H1N1, A/H3N2 and B strain. This vaccine has been administered to patients in a clinical trial, with control patients receiving egg-derived Agrippal™ (which can include formaldehyde and trace amounts of antibiotic). The MDCK-derived vaccine was well tolerated, highly immunogenic (immunologically non-inferior to Agrippal), and met CHMP and CBER criteria for assessment of influenza vaccines. Similar immunogenicity and safety profiles were induced by three different lots of the MDCK-derived vaccine, confirming that the cell culture manufacturing technology is able to generate consistent clinical results.

It will be understood that the invention has been described by way of example only and modifications may be made whilst remaining within the scope and spirit of the invention.

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CLAIMS

1. A vaccine comprising an influenza virus antigen, wherein the vaccine contains no antibiotic, no formaldehyde and no egg-derived materials.
2. A vaccine comprising an influenza virus antigen, wherein the vaccine contains no mercurial preservative, no antibiotic and no formaldehyde.
3. A vaccine comprising an influenza virus antigen, wherein the vaccine contains no mercurial preservative, no antibiotic, no formaldehyde and no egg-derived materials.
4. The vaccine of any preceding claim, having less than 0.1 IU/ml of endotoxin.
5. The vaccine of any preceding claim, having less than 10ng of host cell DNA per 15µg of haemagglutinin.
6. The vaccine of any preceding claim, wherein the antigen is a whole virus antigen.
7. The vaccine of any preceding claim, wherein the antigen is a split virus antigen.
8. The vaccine of any preceding claim, wherein the antigen is a purified surface glycoprotein antigen.
9. The vaccine of any preceding claim, wherein the antigen is a virosome.
10. The vaccine of any preceding claim, including antigen from more than one influenza virus strain.
11. A process for preparing an influenza virus antigen, comprising the steps of: (i) growing influenza virus in a cell culture system, in the absence of egg-derived materials and of antibiotics; (ii) inactivating the influenza viruses grown in step (i), in the absence of formaldehyde; and (iii) preparing a vaccine antigen formulation from the inactivated influenza viruses, in the absence of thimerosal.