(12) STANDARD PATENT (11) Application No. **AU 2007231027 B2**
(19) AUSTRALIAN PATENT OFFICE

(54) Title
Storage of influenza vaccines without refrigeration

(51) International Patent Classification(s)
C07K 14/11 (2006.01) ***A61K 47/26*** (2006.01)
A61K 39/145 (2006.01) ***A61P 31/16*** (2006.01)

(21) Application No: **2007231027** (22) Date of Filing: **2007.03.23**

(87) WIPO No: **WO07/110776**

(30) Priority Data

(31) Number	(32) Date	(33) Country
06251602.6	**2006.03.24**	**EP**

(43) Publication Date: **2007.10.04**
(44) Accepted Journal Date: **2013.10.03**

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(56) Related Art
COENEN et al; VACCINE (2006), vol. 24, no. 4, pages 525-531
HALPERIN, S. A. et al; VACCINE (2002), vol. 20, no. 7-8, pages 1240-1247
VAZQUEZ, N. G. et al; FARMACIA HOSPITALARIA SPAIN (1997), vol. 21, no. 5, pages 283-288
WO 2002/074336 A2

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
4 October 2007 (04.10.2007)

PCT

(10) International Publication Number
WO 2007/110776 A1

(51) International Patent Classification:
A61K 39/145 (2006.01) *A61P 31/16* (2006.01)
A61K 47/26 (2006.01) *C07K 14/11* (2006.01)

(21) International Application Number:
PCT/IB2007/001149

(22) International Filing Date: 23 March 2007 (23.03.2007)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
06251602.6 24 March 2006 (24.03.2006) EP

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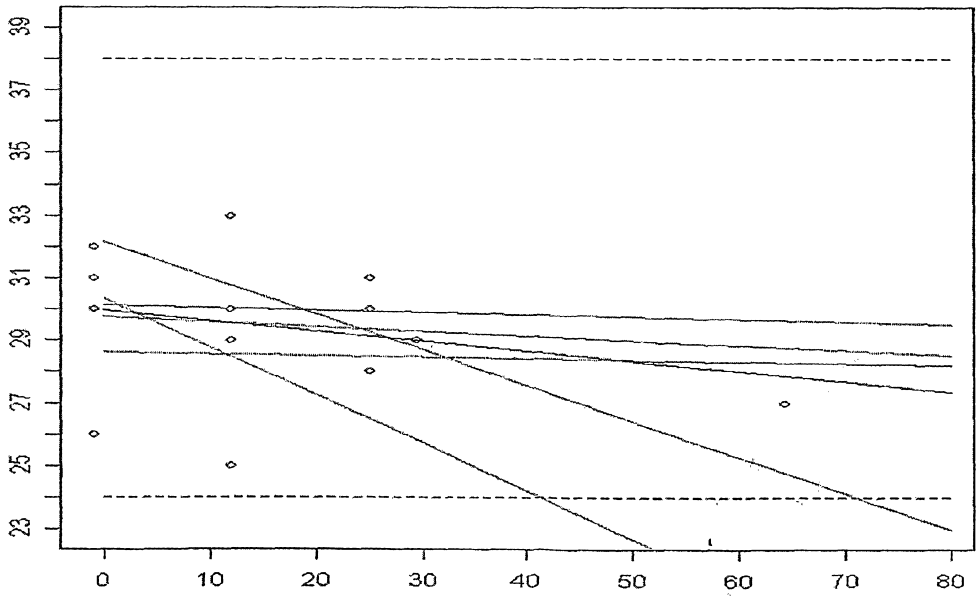
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

[Continued on next page]

(54) Title: STORAGE OF INFLUENZA VACCINES WITHOUT REFRIGERATION



(57) Abstract: Antigens from individual influenza virus strains are not refrigerated before being combined to make multivalent influenza virus vaccines. Moreover, influenza vaccines are not refrigerated between packaging and administration. Thus the need for refrigeration is minimized, and the cold-chain does not have to be maintained between vaccine manufacture and administration.

WO 2007/110776 A1

— *the filing date of the international application is within two months from the date of expiration of the priority period*

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STORAGE OF INFLUENZA VACCINES WITHOUT REFRIGERATION

All documents cited herein are incorporated by reference in their entirety.

TECHNICAL FIELD

This invention is in the field of vaccines for protecting against influenza virus infection, and in particular vaccines that retain efficacy without requiring refrigeration.

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.

The shelf life required for a typical influenza vaccine is 52 weeks. To meet this requirement, a feature common to current influenza vaccines is that they are kept in refrigerated conditions up to the point of administration. These conditions maintain the stability of the protective antigens, including haemagglutinin (HA).

Reference 2 includes an analysis of HA degradation in various vaccine preparations, and reports that HA content can remain within acceptable limits for 78 weeks if stored at 5°C, but that an increase in storage temperature to 25°C (*i.e.* room temperature) increases the degradation rate by at least 6-fold (and up to 24-fold in the worst observed case). While the estimated shelf life of one of the tested vaccines was 104 weeks when stored at 5°C, this decreased to 16 weeks when stored at 25°C, and it was estimated that this vaccine would be unusable if it was exposed to 25°C for >5.3 weeks.

Because of this temperature sensitivity, current influenza vaccine manufacturing, packaging and distribution systems require the cold chain to be maintained.

To avoid the cold chain requirement, various alternative vaccination strategies have been proposed. For instance, references 3 & 4 report the formulation of influenza vaccine as a dry powder. The use of DNA vaccines instead of protein-based vaccines has also been proposed.

It is an object of the invention to provide further and improved influenza vaccines, and processes for their manufacture, which avoid the need to maintain the cold chain, and in particular which avoid the need for the cold chain during distribution and/or on the premises of a final healthcare provider.

DISCLOSURE OF THE INVENTION

According to the invention, influenza vaccines do not have to be refrigerated between packaging and administration. Despite the indications in the prior art, the HA content of influenza vaccines can remain within acceptable limits even when stored at room temperature for at least 6 months.

The invention also allows the avoidance of refrigerated conditions during bulk antigen manufacture after viral growth (*e.g.* during antigen purification) but, in order to avoid the need to change existing approved manufacturing methods, these steps may still be performed under refrigerated conditions, with post-bulk steps (*e.g.* mixing of antigens from different strains, dose filling, storage, distribution)

being performed without refrigeration. One important benefit of the invention is to permit non-refrigerated storage of packaged vaccines by distributors and/or physicians *etc.* Moreover, the invention allows improved patient comfort, as the vaccines are administered at a temperature which is closer to body temperature.

Thus the invention provides a process for distributing a plurality of packaged aqueous influenza vaccines, comprising a step in which the vaccines are transported from a first location to a second location under non-refrigerated conditions. The first and second locations are preferably separated by more than 1 kilometre.

The invention also provides a process for distributing a bulk aqueous influenza vaccine, comprising a step in which the vaccines are moved from a first location to a second location under non-refrigerated conditions. The first and second locations are preferably separated by more than 1 kilometre. The bulk may be a monovalent or a multivalent bulk. The process may include the further step of extracting a unit dose of vaccine from the bulk and placing the unit dose into a container.

Thus the invention provides a process for distributing a plurality of packaged aqueous influenza vaccines, comprising a step in which the vaccines are transported from a first location to a second location, and wherein the vaccines are stored in the second location under non-refrigerated conditions for at least *h* hours. The value of *h* is selected from 12, 18, 24, 36, 48, 60, 72, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1500, 2000, 5000 or more. The second location is preferably a location at which vaccines are administered to patients *e.g.* a clinic, a surgery, *etc.*

The invention also provides a process for distributing an influenza vaccine, wherein the vaccine is moved from a first location to a second location under non-refrigerated conditions, wherein the second location is a site at which a patient can be (and preferably is) vaccinated with the vaccine. The second location can be, for example, a clinic, a healthcare centre, a shopping mall, a patient's home, a patient's workplace, *etc.*

The invention also provides a process for storing an aqueous influenza vaccine, comprising a step in which the vaccine is stored at more than 10°C for more than 10 weeks. The stored vaccine may be a bulk vaccine (monovalent or multivalent) or a packaged vaccine. Where the vaccine is a bulk vaccine, the process may include the further step of extracting a unit dose of vaccine from the bulk and placing the unit dose into a container. Where the bulk is a monovalent bulk, the process may include the further step of combining the monovalent bulk (before, during or after any relevant dilution) with a separate monovalent bulk. Where the vaccine is a packaged vaccine, it is preferably not (i) a trivalent vaccine including strains A/Panama/2007/99 RESVIR-17 reass. and A/New Caledonia/20/99 IVR-116 reass. and B/Yamanashi/166/98; (ii) a trivalent vaccine including strains A/Panama/2007/99 RESVIR-17 reass. and A/New Caledonia/20/99 IVR-116 reass. and B/Guangdong/120/00; or (iii) a trivalent vaccine including strains A/Panama/2007/99 RESVIR-17 reass. and A/New Caledonia/20/99 IVR-116 reass. and B/Shangdong/7/97.

The invention also provides a process for preparing a packaged aqueous vaccine from a vaccine bulk, comprising a step in which a unit dose of vaccine is removed from the bulk and placed into a container under non-refrigerated conditions.

The invention also provides a process for diluting a concentrated influenza antigen bulk, comprising a step in which the concentrated bulk is diluted with an aqueous medium under non-refrigerated conditions. This process gives an antigen preparation with a desired final concentration. The bulk may contain antigens from multiple influenza viruses. As an alternative, it may be a monovalent bulk containing antigen from a single influenza virus strain, in which case the process may include the further step of combining the diluted antigen with antigen from one or more further influenza virus strains, to provide a multivalent composition.

The invention also provides a process for preparing a multivalent influenza vaccine, comprising a step in which an aqueous preparation comprising antigen from a first influenza virus strain is mixed under non-refrigerated conditions with an aqueous preparation comprising antigen from a second influenza virus strain. This process may be used to prepare a bulk vaccine, or it may be used to prepare a packaged vaccine. Preferably this process is used to prepare a trivalent influenza vaccine by mixing antigens from three different influenza virus strains. Where the process is used to prepare a bulk vaccine, the process may include the further step of extracting a unit dose of vaccine from the bulk and placing the unit dose into a container.

The invention also provides a process for inactivating a composition comprising an influenza virus, wherein the process comprises a step in which the composition is mixed with an inactivating agent (*e.g.* with formaldehyde; see further below) under non-refrigerated conditions.

The invention also provides a process for preparing an adjuvanted influenza virus vaccine, comprising a step in which an influenza virus antigen is combined with an adjuvant under non-refrigerated conditions. This process may be used to provide bulk vaccine, and so the process may include the further step of extracting a unit dose of adjuvanted vaccine from the bulk and placing the unit dose into a container. The influenza virus antigen that is combined with the adjuvant is preferably multivalent.

The invention also provides a vaccine obtainable or obtained by these processes.

The invention also provides an aqueous influenza virus vaccine that has been stored under non-refrigerated conditions for at least *h* hours, wherein: (a) *h* is selected from 12, 18, 24, 36, 48, 60, 72, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1500, 2000, 5000 or more; and (b) the vaccine is not (i) a trivalent vaccine including strains A/Panama/2007/99 RESVIR-17 reass. and A/New Caledonia/20/99 IVR-116 reass. and B/Yamanashi/166/98; (ii) a trivalent vaccine including strains A/Panama/2007/99 RESVIR-17 reass. and A/New Caledonia/20/99 IVR-116 reass. and B/Guangdong/120/00; or (iii) a trivalent vaccine including strains A/Panama/2007/99 RESVIR-17 reass. and A/New Caledonia/20/99 IVR-116 reass. and B/Shangdong/7/97.

The invention also provides an aqueous influenza virus vaccine that has been stored under non-refrigerated conditions for at least *h* hours, wherein: (a) *h* is selected from 12, 18, 24, 36, 48, 60, 72, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1500, 2000, 5000 or more; and (b) the vaccine is prepared from influenza viruses grown in cell culture. Unlike vaccines prepared from chicken eggs, therefore, this vaccine can be free from chicken DNA and from egg proteins (such as ovalbumin and ovomucoid), thereby reducing allergenicity.

The invention also provides a kit comprising (a) an aqueous influenza vaccine, and (b) written material indicating that the vaccine can be (i) stored under non-refrigerated conditions and/or (ii) stored at room temperature.

The invention also provides an aqueous vaccine containing haemagglutinin from at least one strain of influenza virus wherein, when the vaccine is stored at 25°C, the rate of degradation of the haemagglutinin is less than 33% per year per strain. With an antigen concentration of 30μg/ml per strain, for instance, the rate of degradation is less than 10μg/ml/year/strain.

The invention also provides an aqueous vaccine containing neuraminidase from at least one strain of influenza virus wherein, when the vaccine is stored at 25°C, the rate of degradation of the neuraminidase is less than 33% per year per strain.

The invention also provides an aqueous vaccine containing both haemagglutinin and neuraminidase from at least one strain of influenza virus wherein, when the vaccine is stored at 25°C, the rate of degradation of both the haemagglutinin and the neuraminidase is less than 33% per year per strain.

The invention also provides the use of an influenza virus antigen in the manufacture of a medicament for administering to a patient by a medical practitioner, wherein the medicament is not refrigerated between the manufacture of the medicament and its being received by a medical practitioner.

The invention also provides the use of an influenza virus antigen in the manufacture of a medicament for administering to a patient by a medical practitioner, wherein the medicament is not refrigerated between being received by the medical practitioner and being administered to the patient.

The invention also provides a process for administering an influenza virus vaccine to a patient wherein, during the 24 hours preceding the administration (*e.g.* preceding injection), the vaccine has been stored under non-refrigerated conditions for at least 12 hours (preferably for at least 18 hours, and more preferably for at least 23 hours *e.g.* for all 24 of the previous 24 hours).

The invention also provides a process for administering an influenza virus vaccine to a patient, wherein the vaccine is administered to the patient after it has been stored under non-refrigerated conditions for at least *h* hours, wherein *h* is selected from 12, 18, 24, 36, 48, 60, 72, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1500, 2000, 5000 or more.

The antigens used in the processes, uses and vaccines of the invention are preferably prepared from viruses grown in cell culture, rather than from viruses grown in eggs, as these antigens have been found to be particularly stable. Without wishing to be bound by theory, the inventors currently

believe that this increased stability relative to egg-grown antigens may have two possible causes: (a) residual egg-derived components (*e.g.* enzymes, such as proteases and/or glycosidases) may be responsible for HA degradation in current vaccines, and so the avoidance of egg as the viral growth substrate can provide a vaccine with better thermal stability; and/or (b) the glycoforms of influenza virus glycoproteins that are obtained in the cell culture, particularly in mammalian cell culture, are more stable than the glycoforms that are obtained in eggs.

Vaccines of the invention may include a detergent *e.g.* a polyoxyethylene sorbitan ester surfactant (known as 'Tweens'), an octoxynol (such as octoxynol-9 (Triton X-100) or t-octylphenoxypolyethoxyethanol), a cetyl trimethyl ammonium bromide ('CTAB'), or sodium deoxycholate, particularly for a split or surface antigen vaccine. Tween 80 may be present at a mass excess relative to HA (*i.e.* more than 1μg per μg of HA), such as between 5 and 25 μg per μg of HA *e.g.* between 10 and 15μg/μg. CTAB may be present at between 0.5 and 2.5 μg per μg of HA *e.g.* between 1.0 and 1.5μg/μg. Tween 80 and CTAB may both be present. The detergent(s) may stabilize influenza virus antigens (such as HA) and prevent their thermal degradation. The presence of Tween 80 (polysorbate 80) in particular may explain the thermal stability seen in the examples below.

Antigen components

The invention uses influenza virus antigens. The antigens will typically be prepared from influenza virions but, as an alternative, antigens can be expressed in a recombinant host and used in purified form. For instance, recombinant haemagglutinin has been used as an antigen *e.g.* expressed in an insect cell line using a baculovirus vector [5,6], as has recombinant neuraminidase [7]. In general, however, antigens will be from virions.

The antigen may take the form of a live virus or, more preferably, an inactivated virus. Chemical means for inactivating a virus include treatment with an effective amount of one or more of the following agents: detergents, formaldehyde (*e.g.* as formalin), β-propiolactone, or UV light. Additional chemical means for inactivation include treatment with 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. The INFLEXAL™ product is a whole virion inactivated vaccine.

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 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. 8-13, *etc.* Splitting of

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, 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, polyoxyethlene 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. The BEGRIVAC™, FLUARIX™, FLUZONE™ and FLUSHIELD™ products are split vaccines.

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 in the art. The FLUVIRIN™, AGRIPPAL™ and INFLUVAC™ products are subunit vaccines.

Influenza antigens can also be presented in the form of virosomes [14] (nucleic acid free viral-like liposomal particles), as in the INFLEXAL V™ and INVAVAC™ products, but it is preferred not to use virosomes with the present invention. Thus, in some embodiments, the influenza antigen is not in the form of a virosome.

The influenza virus may be attenuated. The influenza virus may be temperature-sensitive. The influenza virus may be cold-adapted. These three features are particularly useful when using live virus as an antigen.

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). A large decrease in HA degradation rate, compared to reference 2, has been seen with H1 antigen. Reduced degradation rates have also been seen with H3 antigen, as well as with influenza B virus antigens.

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 [15], with clades 1 and 3 being particularly relevant.

Other strains whose antigens can usefully be included in the compositions are strains which are resistant to antiviral therapy (*e.g.* resistant to oseltamivir [16] and/or zanamivir), including resistant pandemic strains [17].

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.

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.

The influenza virus may be a reassortant strain, and may have been obtained by reverse genetics techniques. Reverse genetics techniques [*e.g.* 18-22] 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 polI promoters, and (b) DNA molecules that encode viral proteins *e.g.* from polII 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 [23-25], 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 [26] 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 26 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.

As an alternative to using polI promoters to encode the viral RNA segments, it is possible to use bacteriophage polymerase promoters [27]. 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.

In other techniques it is possible to use dual polI and polII promoters to simultaneously code for the viral RNAs and for expressible mRNAs from a single template [28,29].

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 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 culture but, in some embodiments, they may be grown on eggs. The current standard method for influenza virus growth uses specific pathogen-free (SPF) embryonated hen eggs, with virus being purified from the egg contents (allantoic fluid). If egg-based viral growth is used then one or more amino acids may be introduced into the allantoid fluid of the egg together with the virus [12].

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 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.* The use of mammalian cells means that vaccines can be free from chicken DNA, as well as being free from egg proteins (such as ovalbumin and ovomucoid), thereby reducing allergenicity.

Preferred mammalian cell lines for growing influenza viruses include: MDCK cells [30-33], derived from Madin Darby canine kidney; Vero cells [34-36], derived from African green monkey (*Cercopithecus aethiops*) kidney; or PER.C6 cells [37], derived from human embryonic retinoblasts. These cell lines are widely available *e.g.* from the American Type Cell Culture (ATCC) collection [38], from the Coriell Cell Repositories [39], or from the European Collection of Cell Cultures (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. 40-42], including avian embryonic stem cells [40,43] and cell lines derived from ducks (*e.g.* duck retina), or from hens. Suitable avian embryonic stem cells, include the EBx cell line derived from chicken embryonic stem cells, EB45, EB14, and EB14-074 [44]. 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 30 discloses a MDCK cell line that was adapted for growth in suspension culture ('MDCK 33016', deposited as DSM ACC 2219). Similarly, reference 45 discloses a MDCK-derived cell line that grows in suspension in serum-free culture ('B-702', deposited as FERM BP-7449). Reference 46 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 47 discloses MDCK cell lines with high susceptibility to infection, including 'MDCK.5F1' cells (ATCC CRL-12042). Any of these MDCK 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 [48]. Absence of herpes simplex viruses is particularly preferred.

Virus may be grown on cells in suspension [30,49,50] 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.

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 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 [51] (*e.g.* 30-36°C) during viral replication.

The method for propagating virus in cultured cells generally includes the steps of inoculating the 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 $TCID_{50}$) 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 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.

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 [65,66], as have higher doses (*e.g.* 3x or 9x doses [52,53]). 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. Dilution to the final desired HA concentration from a concentrated bulk may be performed under non-refrigerated conditions.

For live vaccines, dosing is measured by median tissue culture infectious dose ($TCID_{50}$) rather than HA content, and a $TCID_{50}$ of between 10^6 and 10^8 (preferably between $10^{6.5}$-$10^{7.5}$) per strain is typical.

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) that cause a virus to be highly pathogenic in avian species, as these determinants can otherwise prevent a virus from being grown in eggs..

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.

In some embodiments, particularly where (a) the antigens are prepared from vaccines grown in eggs, (b) the vaccine is an inactivated surface antigen vaccine, and/or (c) the vaccine includes thiomersal, the invention does not relate to the following three vaccines, disclosed in reference 2: (i) a trivalent vaccine including strains A/Panama/2007/99 RESVIR-17 reass. and A/New Caledonia/20/99 IVR-116 reass. and B/Yamanashi/166/98; (ii) a trivalent vaccine including strains A/Panama/2007/99 RESVIR-17 reass. and A/New Caledonia/20/99 IVR-116 reass. and B/Guangdong/120/00; or (iii) a trivalent vaccine including strains A/Panama/2007/99 RESVIR-17 reass. and A/New Caledonia/20/99 IVR-116 reass. and B/Shangdong/7/97.

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 [54,55]. 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 [56]; immunoassay methods, such as the Threshold™ System [57]; and quantitative PCR [58]. 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 [57]. 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 59.

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 60 & 61, 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 [62].

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

Storage conditions and transport

Where a vaccine or an antigen composition is said not to be refrigerated (or similar wording), this means that it is not stored under conditions such that it equilibrates to reach a temperature ≤8°C. Thus a composition that is transiently placed into a refrigerator, but not for a sufficiently long period for its overall temperature to drop to 8°C or lower, has not been refrigerated. In preferred embodiments, however, the invention permits the avoidance any contact with refrigerated conditions, even for transient periods.

Where the invention involves transport between two locations, these are preferably more than *n* kilometres apart, where *n* is selected from 1, 2, 3, 4, 5, 10, 20, 25, 30, 40, 50, 100 or more.

Where a composition is stored at more than 10°C, it may be stored at ≥11°C, ≥12°C, ≥13°C, ≥14°C, ≥15°C, ≥16°C, ≥17°C, ≥18°C, ≥19C, ≥20°C, ≥21°C, ≥22°C, ≥23°C, ≥24°C. Typically, it will be stored at below 40°C *e.g.* ≤39°C, ≤38°C, ≤37°C, ≤36°C, ≤35°C, ≤34°C, ≤33°C, ≤32°C, ≤31°C, ≤30°C, ≤29°C, ≤28°C, ≤27°C, ≤26°C. A typical storage temperature will be at room temperature *e.g.* between 18°C and 23°C *e.g.* 20±1°C.

Where a composition is stored under non-refrigerated conditions for more than 10 weeks, it may be stored for ≥11 weeks, ≥12 weeks, ≥13 weeks, ≥14 weeks, ≥15 weeks, ≥16 weeks, ≥17 weeks, ≥18 weeks, ≥19 weeks, ≥20 weeks, ≥25 weeks, ≥26 weeks, ≥30 weeks, ≥35 weeks, ≥40 weeks. Storage for less than 1 year is usual.

Vaccines may be stored out of direct light *e.g.* in the dark.

Where a vaccine has a rate of haemagglutinin degradation of less than 33% per year per strain, it is preferably ≤32%, ≤31%, ≤30%, ≤29%, ≤28%, ≤27%, ≤26%, ≤25%, ≤24%, ≤23%, ≤22%, ≤21%, ≤20%, ≤19%, ≤18%, ≤17%, ≤16%, ≤15%, ≤14%, ≤13%, ≤12%, ≤11%, ≤10%, ≤9%, ≤8%, ≤7%, ≤6%, ≤5% or lower. The degradation is preferably determined by the ICH (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use) Guideline Q1A(R2), entitled "Stability Testing of New Drug Substances and Products", including the relevant statistical analysis.

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 63 & 64, aluminum hydroxide was used, and in reference 65, a mixture of aluminum hydroxide and aluminum phosphate was used. Reference 66 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:

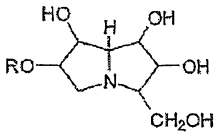
- A mineral-containing composition, including calcium salts and aluminum salts (or mixtures thereof). Calcium salts include calcium phosphate (*e.g.* the "CAP" particles disclosed in ref. 67). Aluminum salts include hydroxides, phosphates, sulfates, *etc.*, with the salts taking any suitable form (*e.g.* gel, crystalline, amorphous, *etc.*). Adsorption to these salts is preferred. The mineral containing compositions may also be formulated as a particle of metal salt [68]. Aluminum salt adjuvants are described in more detail below.
- Cytokine-inducing agents (see in more detail below).
- Saponins [chapter 22 of ref. 104], 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 *Quillaia saponaria* Molina tree have been widely studied as adjuvants. Saponin can also be commercially obtained from *Smilax ornata* (sarsaprilla), *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. 69. It is possible to use fraction A of Quil A together with at least one other adjuvant [70]. Saponin formulations may also comprise a sterol, such as cholesterol [71]. Combinations of saponins and cholesterols can be used to form unique particles called immunostimulating complexs (ISCOMs) [chapter 23 of ref. 104]. 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. 71-73. Optionally, the ISCOMS may be devoid of additional detergent [74]. 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 [75]. A review of the development of saponin based adjuvants can be found in refs. 76 & 77.

- 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 [78]. The use of detoxified ADP-ribosylating toxins as mucosal adjuvants is described in ref. 79 and as parenteral adjuvants in ref. 80.
- Bioadhesives and mucoadhesives, such as esterified hyaluronic acid microspheres [81] or chitosan and its derivatives [82].
- 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).
- Liposomes (Chapters 13 & 14 of ref. 104). Examples of liposome formulations suitable for use as adjuvants are described in refs. 83-85.
- Oil-in-water emulsions (see in more detail below).
- Polyoxyethylene ethers and polyoxyethylene esters [86]. Such formulations further include polyoxyethylene sorbitan ester surfactants in combination with an octoxynol [87] as well as polyoxyethylene alkyl ethers or ester surfactants in combination with at least one additional non-ionic surfactant such as an octoxynol [88]. Preferred polyoxyethylene ethers are selected from the following group: polyoxyethylene-9-lauryl ether (laureth 9), polyoxyethylene-9-steoryl ether, polyoxytheylene-8-steoryl ether, polyoxyethylene-4-lauryl ether, polyoxyethylene-35-lauryl ether, and polyoxyethylene-23-lauryl ether.
- Muramyl peptides, such as N-acetylmuramyl-L-threonyl-D-isoglutamine ("thr-MDP"), N-acetyl-normuramyl-L-alanyl-D-isoglutamine (nor-MDP), N-acetylglucsaminyl-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").

- 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 [89].
- Methyl inosine 5'-monophosphate ("MIMP") [90].
- A polyhydroxlated pyrrolizidine compound [91], such as one having formula:



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-di*epi*-casuarine, *etc.*

- A gamma inulin [92] 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 104 & 105.

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 93-100 *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.

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 5μm 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:

- 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' [101-103], as described in more detail in Chapter 10 of ref. 104 and chapter 12 of ref. 105. The MF59 emulsion advantageously includes citrate ions *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. 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.

- 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 three components at a mass ratio of about 75:11:10 (*e.g.* 750μg/ml polysorbate 80, 110μg/ml Triton X-100 and 100μg/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.

- An emulsion of squalane, 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 [106] (0.05-1% Thr-MDP, 5% squalane, 2.5% Pluronic L121 and 0.2% polysorbate 80). It can also be used without the Thr-MDP, as in the "AF" adjuvant [107] (5% squalane, 1.25% Pluronic L121 and 0.2% polysorbate 80). Microfluidisation is preferred.

- 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 108, 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 109, produced by addition of aliphatic amine to desacylsaponin via the carboxyl group of glucuronic acid), dimethyidioctadecylammonium 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) [110].

- 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) [110].

- An emulsion in which a saponin (*e.g.* QuilA or QS21) and a sterol (*e.g.* a cholesterol) are associated as helical micelles [111].

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 [112]. They also have antioxidant properties that may help to stabilize the emulsions [113]. 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 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 [11].

Cytokine-inducing agents

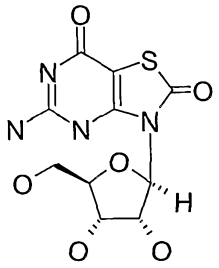
Cytokine-inducing agents for inclusion in compositions of the invention are able, when administered to a patient, to elicit the immune system to release cytokines, including interferons and interleukins. Cytokine responses are known to be involved in the early and decisive stages of host defense against influenza infection [114]. 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.

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 form 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 real-time PCR. For example, reference 115 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

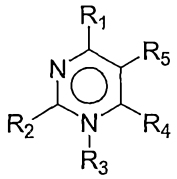
responses from spontaneous responses, but that intracytoplasmic cytokine detection by flow cytometry was the most efficient method to detect re-stimulating effects.

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.
- 3-O-deacylated monophosphoryl lipid A ('3dMPL', also known as 'MPL™') [116-119].
- An imidazoquinoline compound, such as Imiquimod ("R-837") [120,121], Resiquimod ("R-848") [122], and their analogs; and salts thereof (*e.g.* the hydrochloride salts). Further details about immunostimulatory imidazoquinolines can be found in references 123 to 127.
- A thiosemicarbazone compound, such as those disclosed in reference 128. Methods of formulating, manufacturing, and screening for active compounds are also described in reference 128. 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 129. Methods of formulating, manufacturing, and screening for active compounds are also described in reference 129. The thiosemicarbazones are particularly effective in the stimulation of human peripheral blood mononuclear cells for the production of cytokines, such as TNF-α.
- 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 130 to 132; (f) a compound having the formula:

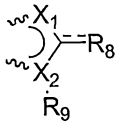


wherein:

R_1 and R_2 are each independently H, halo, $-NR_aR_b$, -OH, C_{1-6} alkoxy, substituted C_{1-6} alkoxy, heterocyclyl, substituted heterocyclyl, C_{6-10} aryl, substituted C_{6-10} aryl, C_{1-6} alkyl, or substituted C_{1-6} alkyl;

R_3 is absent, H, C_{1-6} alkyl, substituted C_{1-6} alkyl, C_{6-10} aryl, substituted C_{6-10} aryl, heterocyclyl, or substituted heterocyclyl;

R_4 and R_5 are each independently H, halo, heterocyclyl, substituted heterocyclyl, $-C(O)-R_d$, C_{1-6} alkyl, substituted C_{1-6} alkyl, or bound together to form a 5 membered ring as in R_{4-5}:



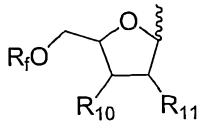
R_{4-5}

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

X_1 and X_2 are each independently N, C, O, or S;

R_8 is H, halo, -OH, C_{1-6} alkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, -OH, $-NR_aR_b$, $-(CH_2)_n-O-R_c$, $-O-(C_{1-6}$ alkyl), $-S(O)_pR_e$, or $-C(O)-R_d$;

R_9 is H, C_{1-6} alkyl, substituted C_{1-6} alkyl, heterocyclyl, substituted heterocyclyl or R_{9a}, wherein R_{9a} is:



R_{9a}

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

R_{10} and R_{11} are each independently H, halo, C_{1-6} alkoxy, substituted C_{1-6} alkoxy, $-NR_aR_b$, or -OH;

each R_a and R_b is independently H, C_{1-6} alkyl, substituted C_{1-6} alkyl, $-C(O)R_d$, C_{6-10} aryl;

each R_c is independently H, phosphate, diphosphate, triphosphate, C_{1-6} alkyl, or substituted C_{1-6} alkyl;

each R_d is independently H, halo, C_{1-6} alkyl, substituted C_{1-6} alkyl, C_{1-6} alkoxy, substituted C_{1-6} alkoxy, $-NH_2$, $-NH(C_{1-6}$ alkyl), -NH(substituted C_{1-6} alkyl), $-N(C_{1-6}$ alkyl)$_2$, -N(substituted C_{1-6} alkyl)$_2$, C_{6-10} aryl, or heterocyclyl;

each R_e is independently H, C_{1-6} alkyl, substituted C_{1-6} alkyl, C_{6-10} aryl, substituted C_{6-10} aryl, heterocyclyl, or substituted heterocyclyl;

each R_f is independently H, C_{1-6} alkyl, substituted C_{1-6} 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 a pharmaceutically acceptable salt of the tautomer.

- Loxoribine (7-allyl-8-oxoguanosine) [133].
- Compounds disclosed in reference 134, including: Acylpiperazine compounds, Indoledione compounds, Tetrahydraisoquinoline (THIQ) compounds, Benzocyclodione compounds, Aminoazavinyl compounds, Aminobenzimidazole quinolinone (ABIQ) compounds [135,136], Hydrapthalamide compounds, Benzophenone compounds, Isoxazole compounds, Sterol compounds, Quinazilinone compounds, Pyrrole compounds [137], Anthraquinone compounds, Quinoxaline compounds, Triazine compounds, Pyrazalopyrimidine compounds, and Benzazole compounds [138].
- A polyoxidonium polymer [139,140] or other N-oxidized polyethylene-piperazine derivative.
- Compounds disclosed in reference 141.
- An aminoalkyl glucosaminide phosphate derivative, such as RC-529 [142,143].
- A CD1d ligand, such as an α-glycosylceramide [144-151] (*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 152 and 153.
- 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

  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

  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](methyl)amino]ethanol

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

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

  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

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

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. 154. 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/analogs such as phosphorothioate modifications and can be double-stranded or (except for RNA) single-stranded. References 155, 156 and 157 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. 158-163. A CpG sequence may be directed to TLR9, such as the motif GTCGTT or TTCGTT [164]. 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 a CpG-B ODN. CpG-A and CpG-B ODNs are discussed in refs. 165-167. 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 164 & 168-170. A useful CpG adjuvant is CpG7909, also known as ProMune™ (Coley Pharmaceutical Group, Inc.).

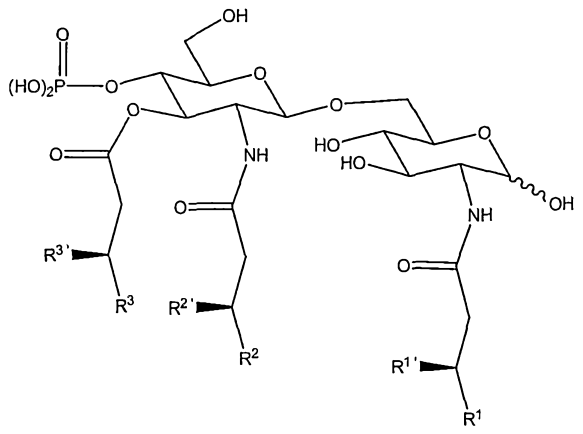
As an alternative, or in addition, to using CpG sequences, TpG sequences can be used [171]. These oligonucleotides may be free from unmethylated CpG motifs.

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. 171), 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. 171), 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. 172). Preparation of 3dMPL was originally described in reference 173.

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-CH_2-$CR^1R^{1'}$. The group attached to carbon 2' has formula -NH-CO-CH_2-$CR^2R^{2'}$. The group attached to carbon 3' has formula -O-CO-CH_2-$CR^3R^{3'}$. A representative structure is:

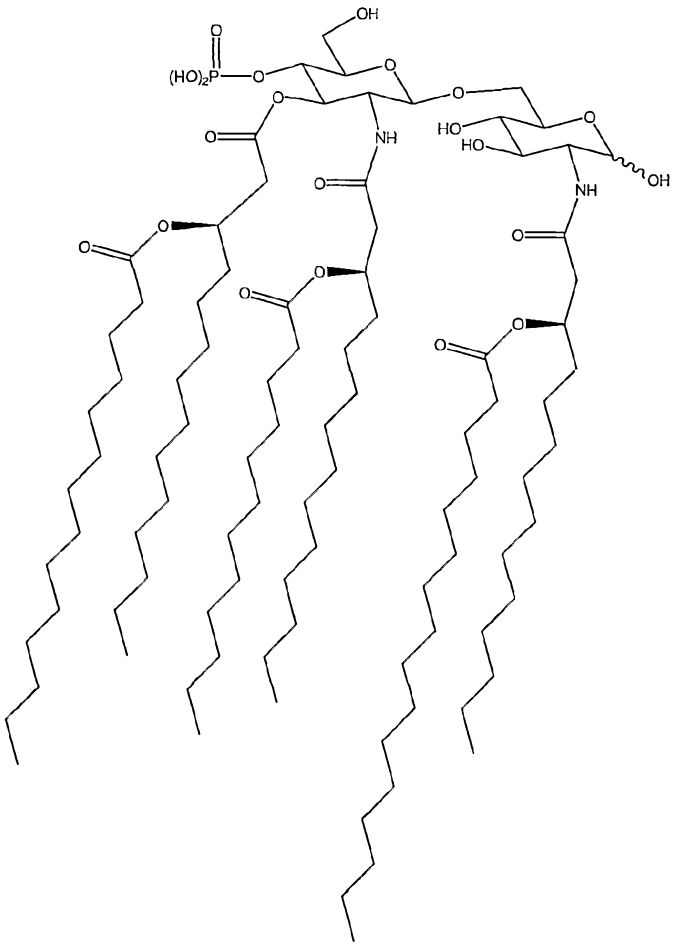


Groups R^1, R^2 and R^3 are each independently $-(CH_2)_n-CH_3$. The value of *n* is preferably between 8 and 16, more preferably between 9 and 12, and is most preferably 10.

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.

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 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.* ≥20%, ≥30%, ≥40%, ≥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 [174]. 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.* ≥60%, ≥70%, ≥80%, ≥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.

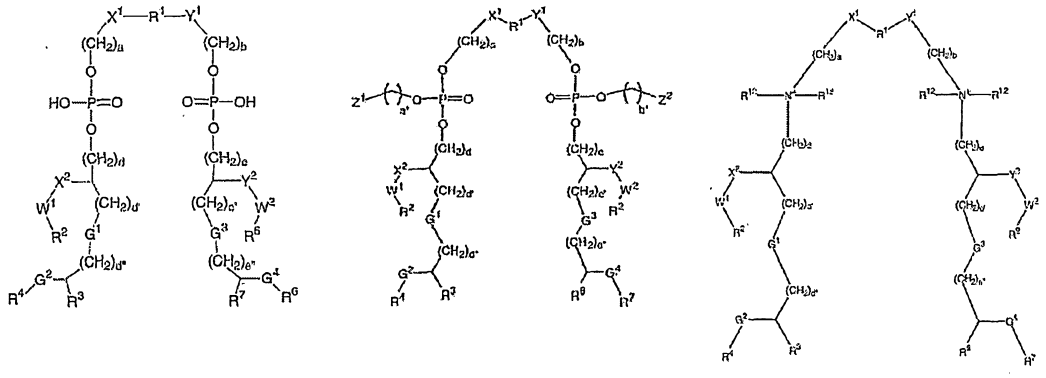
The 3dMPL can be used on its own, or in combination with one or more further compounds. For example, it is known to use 3dMPL in combination with the QS21 saponin [175] (including in an oil-in-water emulsion [176]), with an immunostimulatory oligonucleotide, with both QS21 and an immunostimulatory oligonucleotide, with aluminum phosphate [177], with aluminum hydroxide [178], or with both aluminum phosphate and aluminum hydroxide.

Fatty adjuvants

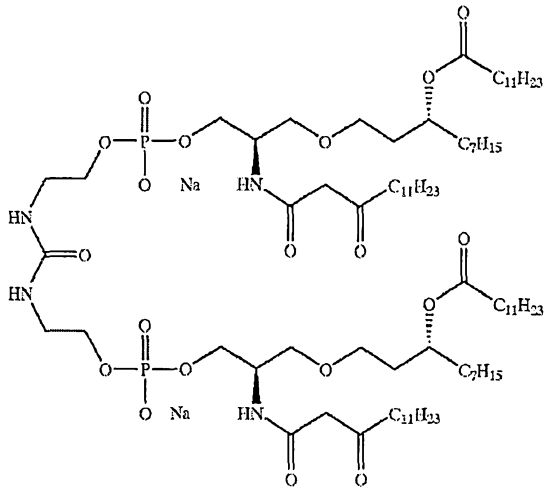
Fatty adjuvants that can be used with the invention include the oil-in-water emulsions described above, and also include, for example:

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

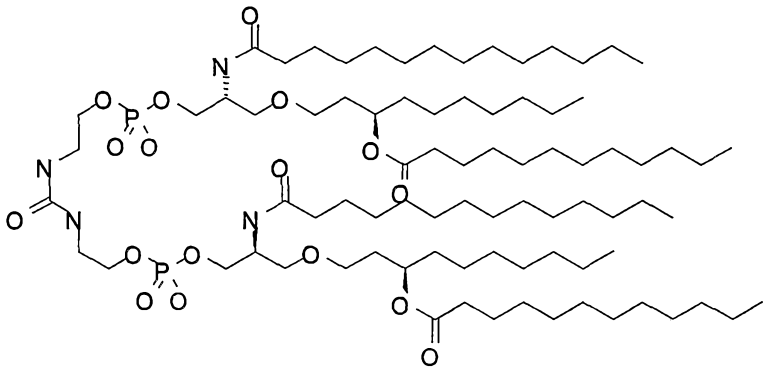
I II III



as defined in reference 179, 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.*:

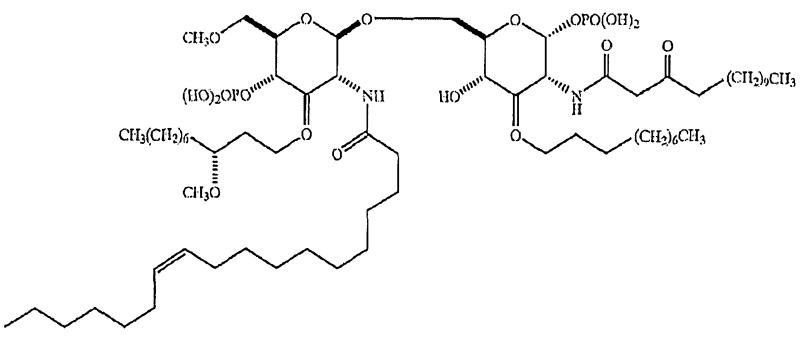


ER804057



ER-803022:

- Derivatives of lipid A from *Escherichia coli* such as OM-174 (described in refs. 180 & 181).
- A formulation of a cationic lipid and a (usually neutral) co-lipid, such as aminopropyl-dimethyl-myristoleyloxy-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 [182].
- 3-O-deacylated monophosphoryl lipid A (see above).
- Compounds containing lipids linked to a phosphate-containing acyclic backbone, such as the TLR4 antagonist E5564 [183,184]:



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 104). 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. 104]. 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 PO_4/Al molar ratio between 0.3 and 1.2. Hydroxyphosphates can be distinguished from strict $AlPO_4$ 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. 104].

The PO_4/Al^{3+} 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 PO_4/Al molar ratio between 0.84 and 0.92, included at 0.6mg Al^{3+}/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 [65]. 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 173, and the product has been manufactured and sold by Corixa Corporation under the name MPL™. Further details can be found in refs 116 to 119.

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 185.

Compositions will generally be in aqueous form.

The composition may include preservatives such as thiomersal or 2-phenoxyethanol. It is preferred, however, that the vaccine should be substantially free from (*i.e.* less than 5μg/ml) mercurial material *e.g.* thiomersal-free [11,186]. Vaccines containing no mercury are more preferred. Preservative-free vaccines are particularly 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 [187], but keeping osmolality in this range is nevertheless preferred.

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 non-pyrogenic *e.g.* containing <1 EU (endotoxin unit, a standard measure) per dose, and preferably <0.1 EU per dose. The composition is preferably gluten free.

The vaccine may include residual components in trace amounts, such as antibiotics (*e.g.* neomycin, kanamycin, polymyxin B).

The composition may include material for a single immunisation, or may include material for multiple immunisations (*i.e.* a 'multidose' composition). The inclusion of a preservative is preferred in multidose arrangements. As an alternative (or in addition) to including a preservative in multidose compositions, the compositions 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.

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.

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

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 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 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. 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"™.

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.

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 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.

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

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) [188]. 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.

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 [189-191], oral [192], intradermal [193,194], transcutaneous, transdermal [195], *etc.*

Vaccines prepared according to the invention may be used to treat both children and adults. Influenza 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, 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.

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) ≥70% seroprotection; (2) ≥40% seroconversion; and/or (3) a GMT increase of ≥2.5-fold. In elderly (>60 years), these criteria are: (1) ≥60% seroprotection; (2) ≥30% seroconversion; and/or (3) a GMT increase of ≥2-fold. These criteria are based on open label studies with at least 50 patients.

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 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) 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 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 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, 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.

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, $x \pm 10\%$.

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.*

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 encaphalopathies (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.

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.*

BRIEF DESCRIPTION OF DRAWINGS

Figures 1 to 7 show HA levels (μg/mL) measured over time (months for 2-8°C; days for 37°C) for the following samples:

	Influenza A		Influenza B		Temp (°C)
Fig	New Caledonia	Panama	Guandong	Shangdong	
1	X				2-8
2		X			2-8
3			X		37
4	X				37
5	X	X	X		2-8
6	X	X	X		2-8
7	X	X		X	37

Figure 8 extrapolates HA content (μg/ml) over a 80 week period for B/Jiangsu at 23-27°C. Data were statistically evaluated with a separate or homogeneous model, and the Figure shows the regression line with 95% confidence limits. A comparison with the influenza B virus data from reference 2 data shows that the present materials have a longer shelf life.

MODES FOR CARRYING OUT THE INVENTION

Various influenza virus strains were grown on a MDCK cell substrate [30]: A/Panama (H3N2); A/New Caledonia (HlNl); A/Wyoming (H3N2); A/Wellington (H3N2); B/Shangdong; B/Guangdong; and B/Jiangsu. Strains were grown separately and monovalent antigen bulks were prepared from each. Strains were selected and combined to give final trivalent lots for clinical use. The final product included Tween 80 (polysorbate 80) detergent (<25 μg/μg HA) and was mercury-free. Residual CTAB from virus disruption was also present.

The monovalent bulks and final trivalent lots were stored under refrigerated conditions (2-8°C) or non-refrigerated conditions (23-27°C), and the HA content was determined at various timepoints by SRID. The HA concentration was different for each monovalent bulk, but in final trivalent material the specified starting HA levels (time zero) were 15μg per strain per dose, which is 30μg/ml/strain.

Figures 1 to 7 show examples of HA levels of various monovalent or trivalent materials measured over time when stored at various temperatures.

The vaccines in Figures 1-7 showed excellent stability at 2-8°C for 12 months and at 37°C for 4 weeks. Moreover, for all lots, including those for which data are not shown, stability requirements were met at each measured timepoint, and no lots failed to meet the stability specification. Thus all

the monovalent bulks could be stored at 2-8°C for 12 months or at 37°C for 4 weeks, and the same is true for the final trivalent products. When stored at 2-8°C, the data suggest a 39 month shelf-life.

The following table shows HA levels in various monovalent vaccine lots stored at 23-27°C for up to 15 months. In no cases was a HA level seen below 80% of the starting concentration:

		Time (months) at 23-27°C				
Batch	**Strain**	0	3	6	7	15
522011011	Jiangsu	28	27	n.a.	n.a.	n.a.
522011011	New Caledonia	28	32	n.a.	n.a.	n.a.
522011011	New York	31	26	n.a.	n.a.	n.a.
522008011	Jiangsu	31	33	**28**	n.a.	n.a.
522008011	New Caledonia	30	26	**25**	n.a.	n.a.
522008011	New York	31	27	**26**	n.a.	n.a.
522008012	Jiangsu	32	25	**28**	n.a.	n.a.
522008012	New Caledonia	29	25	**26**	n.a.	n.a.
522008012	New York	31	26	**28**	n.a.	n.a.
522009011	Jiangsu	25	29	n.a.	n.a.	n.a.
522009011	New Caledonia	26	24	n.a.	n.a.	n.a.
522009011	New York	29	27	n.a.	n.a.	n.a.
522007011	Jiangsu	26	30	31	n.a.	27
522007011	New Caledonia	28	31	30	n.a.	27
522007011	Wyoming	32	30	30	n.a.	30
522004011 backup	Jiangsu	30	30	31	29	n.a.
522004011 backup	New Caledonia	30	29	29	25	n.a.
522004011 backup	Wyoming	30	29	24	27	n.a.
522006011 backup	Jiangsu	30	29	30	29	n.a.
522006011 backup	New Caledonia	30	30	29	27	n.a.
522006011 backup	Wyoming	30	29	24	27	n.a.
Test Blending 30505	Jiangsu	29	26	n.a.	n.a.	n.a.
Test Blending 30505	New Caledonia	27	27	n.a.	n.a.	n.a.
Test Blending 30505	Wellington	29	30	n.a.	n.a.	n.a.

Based on HA measurements over time, it is possible to extrapolate HA levels. This can give an expected shelf-life *i.e.* the period before HA concentration drops below 24μg/ml, or 80% of the starting amount). The expected shelf-life for various clinical trivalent materials is given in the following table, measured in months for storage at 2-8°C. Viral strains were: (N) A/New Caledonia; (P) A/Panama; (G) B/Guangdong:

Lot A			**Lot B**		**Lot C**		
N	**P**	**G**	**N**	**P**	**N**	**P**	**G**
≥39	≥30	≥36	≥33	≥24	≥24	≥24	≥24/15

An expected shelf life of at least 15 months is seen for all clinical materials. A shelf life of 15 months was also estimated for material prepared for pre-clinical toxicity testing.

The HA data were compared to the data in reference 2 to provide a HA degradation rate and an estimated shelf life. Due to changes in influenza season, however, direct strain-to-strain comparisons were not possible in all cases.

Figure 8 shows one example of a statistical comparison between the reference 2 study and the present invention.

For storage at 23-27°C, the data in reference 2 show HA degradation occurring at between 11.37 μg/ml/year and 33.71 μg/ml/year. In contrast, the test vaccines showed HA degradation occurring at much lower rates: between 1.71 μg/ml/year and 4.52 μg/ml/year. For the A/New Caledonia strain in particular, the rate observed in reference 2 was 11.37μg/ml/year whereas the rate observed in the test vaccines was 2.67μg/ml/year. Degradation rates were also lower for vaccines stored at 4°C.

	2-8°C			23-27°C	
Strain	**Ref. 2**	**Lot D**	**Lot E**	**Ref. 2**	**Lot E**
A/New Caledonia	-1.57	-0.65	≥0	-11.37	-2.67
A/Panama	-4.90	-2.93	–	-31.05	–
B/Guangdong	-2.71	≥-0.13	–	-33.71	–
B/Jiangsu	–	–	≥0	–	-1.71
A/Wyoming	–	–	-2.96	–	-4.52

The data indicated that the trivalent mixtures could have a shelf life of up to 15 months even at 23-27°C, which is much longer than suggested in reference 2. At 4°C, the shelf life could be up to 42-45 months:

Even when trivalent vaccine was stored at 40°C, a shelf-life of about 6 months for the B/Jiangsu component was estimated. At a lower storage temperature (25°C) this rises to at least 9 months, and possibly 18 months or more.

Possible explanations for the enhanced stability, compared to reference 2, include: (i) the present vaccines were prepared from mammalian cell culture, rather than from eggs, and the degradation seen in reference 2 may have been due to residual egg derived components (*e.g.* enzymes, such as proteases and/or glycosidases) and/or to differences in glycosylation; or (ii) the higher level of Tween 80 present in the present vaccines, which may stabilize HA.

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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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A process for producing one or a plurality of aqueous influenza vaccines, each vaccine comprising hemagglutinin from at least one strain of influenza virus grown in cell culture and polysorbate 80 in relative amounts of more than 1 μg polysorbate 80 per μg of hemagglutinin, said process comprising storing the vaccine(s) at a storage temperature of more than 10°C for more than 14 weeks, and wherein a rate of degradation of hemagglutinin in said vaccine(s) is less than 33% per year per strain of influenza virus during the storage.

2. A process for distributing a plurality of packaged aqueous influenza vaccines, each vaccine comprising hemagglutinin from at least one strain of influenza virus grown in cell culture and polysorbate 80 in relative amounts of more than 1 μg polysorbate 80 per μg of hemagglutinin, said process comprising transporting the vaccines from a first location to a second location, and storing the vaccines in the second location under non-refrigerated conditions for at least 1500 hours, wherein a rate of degradation of hemagglutinin in said vaccines is less than 33% per year per strain of influenza virus during the storage.

3. A process for distributing a bulk aqueous influenza vaccine comprising hemagglutinin from at least one strain of influenza virus grown in cell culture and polysorbate 80 in relative amounts of more than 1 μg polysorbate 80 per μg of hemagglutinin, said process comprising moving the bulk aqueous influenza vaccine from a first location to a second location under non-refrigerated conditions and storing the bulk aqueous influenza vaccine, wherein a rate of degradation of hemagglutinin in said vaccine is less than 33% per year per strain of influenza virus during the storage.

4. A process for distributing a plurality of packaged aqueous influenza vaccines, each vaccine comprising hemagglutinin from at least one strain of influenza virus grown in cell culture and polysorbate 80 in relative amounts of more than 1 μg polysorbate 80 per μg of hemagglutinin, said process comprising transporting the vaccines from a first location to a second location under non-refrigerated conditions and storing the vaccines, wherein a rate of degradation of hemagglutinin in said vaccines is less than 33% per year per strain of influenza virus during the storage.

5. A process for preparing a packaged aqueous aqueous influenza vaccine from a bulk aqueous influenza vaccine comprising hemagglutinin from at least one strain of influenza virus grown in cell culture and polysorbate 80 in relative amounts of more than 1 μg polysorbate 80 per μg of hemagglutinin, said process comprising removing a unit dose of vaccine from the bulk aqueous influenza vaccine and placing the unit dose into a container under non-refrigerated conditions and storing the container comprising the unit dose, wherein a rate of degradation of hemagglutinin is less than 33% per year per strain of influenza virus during the storage.

6. A process for diluting a concentrated influenza antigen bulk comprising hemagglutinin from at least one strain of influenza virus grown in cell culture, said process comprising diluting the

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concentrated influenza antigen bulk with an aqueous medium comprising polysorbate 80 under non-refrigerated conditions to produce a diluted influenza antigen comprising more than 1 μg polysorbate 80 per μg of hemagglutinin, and storing the diluted influenza antigen,

wherein a rate of degradation of hemagglutinin is less than 33% per year per strain of influenza virus during the storage,

and wherein:

(i) said bulk is a monovalent bulk or multivalent bulk; and

(ii) optionally when the bulk is a monovalent bulk further comprising combining the diluted antigen with antigen from one or more further influenza virus strains to provide a multivalent composition.

7. A process for diluting a concentrated influenza antigen bulk comprising hemagglutinin from at least one strain of influenza virus grown in cell culture and polysorbate 80 in relative amounts of more than 1 μg polysorbate 80 per μg of hemagglutinin, said process comprising diluting the concentrated influenza antigen bulk with an aqueous medium under non-refrigerated conditions to produce a diluted influenza antigen and storing the diluted influenza antigen,

wherein a rate of degradation of hemagglutinin is less than 33% per year per strain of influenza virus during the storage,

and wherein:

(i) said bulk is a monovalent bulk or multivalent bulk; and

(ii) optionally when the bulk is a monovalent bulk further comprising combining the diluted antigen with antigen from one or more further influenza virus strains to provide a multivalent composition.

8. A process for preparing a multivalent influenza vaccine, said process comprising:

(i) mixing aqueous antigen preparations under non-refrigerated conditions, each antigen preparation comprising antigen from a different influenza virus strain, wherein at least one antigen preparation comprises hemagglutinin from an influenza virus strain grown in cell culture, and wherein the multivalent influenza vaccine produced by said mixing comprises more than 1 μg polysorbate 80 per μg of hemagglutinin; and

(ii) storing the multivalent influenza vaccine, wherein a rate of degradation of hemagglutinin is less than 33% per year per strain of influenza virus when said multivalent influenza vaccine during the storage.

9. The process according to claim 8, comprising mixing antigens from three strains of influenza to produce a trivalent influenza vaccine.

10. A process for inactivating a composition comprising hemagglutinin from an influenza virus strain grown in cell culture and having more than 1 μg polysorbate 80 per μg of influenza virus hemagglutinin, said composition also comprising an influenza virus, wherein the process comprises mixing the composition with an inactivating agent under non-refrigerated

conditions to thereby inactivate the influenza virus, and storing the composition, wherein the rate of degradation of hemagglutinin is less than 33% per year per strain of influenza virus during the storage.

11. A process for preparing an adjuvanted influenza virus vaccine, said process comprising combining an adjuvant with an influenza virus antigen preparation comprising hemagglutinin from an influenza virus strain grown in cell culture and having more than 1 μg polysorbate 80 per μg of influenza virus hemagglutinin under non-refrigerated conditions to produce an adjuvanted influenza virus vaccine, and storing the adjuvanted influenza virus vaccine, wherein a rate of degradation of hemagglutinin is less than 33% per year per strain of influenza virus during the storage.

12. A stored aqueous influenza vaccine comprising hemagglutinin from at least one strain of influenza virus grown in cell culture and polysorbate 80 in relative amounts of more than 1 μg polysorbate 80 per μg of hemagglutinin, wherein said vaccine has been stored under non-refrigerated conditions for at least 100 hours such that a rate of degradation of hemagglutinin in said vaccine is less than 33% per year per strain of influenza virus during the storage.

13. A stored aqueous influenza vaccine comprising hemagglutinin from at least one strain of influenza virus grown in cell culture and polysorbate 80 in relative amounts of more than 1 μg polysorbate 80 per μg of hemagglutinin, wherein when said vaccine is stored at 25°C a rate of degradation of hemagglutinin in said vaccine is less than 33% per year per strain of influenza virus during the storage.

14. The stored aqueous influenza vaccine according to claim 12 or 13 when stored for at least 300 hours, wherein said vaccine is not: (i) a trivalent vaccine comprising strains A/Panama/2007/99 RESVIR-17 reass. and A/New Caledonia/20/99 IVR-116 reass. and B/Yamanashi/166/98; (ii) a trivalent vaccine comprising strains A/Panama/2007/99 RESVIR-17 reass. and A/New Caledonia/20/99 IVR-116 reass. and B/Guangdong/120/00; or (iii) a trivalent vaccine including strains A/Panama/2007/99 RESVIR-17 reass. and A/New Caledonia/20/99 IVR-116 reass. and B/Shangdong/7/97.

15. The stored aqueous vaccine according to any one of claims 12 to 14, wherein the vaccine is:
    (a) free from chicken DNA, ovalbumin and ovomucoid; and/or
    (b) free from mercury and suitable for administration to a human; and/or
    (c) a split virion vaccine, a whole virion vaccine, a live virus vaccine or a virosome vaccine.

16. The stored aqueous influenza vaccine according to any one of claims 12 to 15 when obtained by a method comprising the process according to any one of claims 1 to 11.

17. The process according to any one of claims 1 to 11 or the stored influenza vaccine according to any one of claims 12 to 16, wherein the cell culture is performed on a cell line selected from

the group consisting of: MDCK; CHO; 293T; BHK; Vero; MRC-5; PER.C6; and WI-38 cell lines.

18. The process according to any one of claims 1 to 11 or 17 or the stored influenza vaccine according to any one of claims 12 to 17, wherein the cell culture is performed using MDCK 33016 cells.

19. The process according to any one of claims 1 to 11 or 17 or 18 or the stored influenza vaccine according to any one of claims 12 to 18, wherein the vaccine or hemagglutinin or other antigen thereof is prepared from an inactivated virus or a split virus or wherein the vaccine comprises a purified surface glycoprotein.

20. The process according to any one of claims 1 to 11 or any one of claims 17 to 19 or the stored influenza vaccine according to any one of claims 12 to 19, wherein the vaccine comprises between 5 µg polysorbate 80 per µg hemagglutinin and 25 µg polysorbate 80 per µg hemagglutinin.

21. The process according to any one of claims 1 to 11 or any one of claims 17 to 20 or the stored influenza vaccine according to any one of claims 12 to 20, wherein the vaccine comprises an adjuvant.

22. The process or stored influenza vaccine according to claim 21, whereint he adjuvant is an oil-in-water emulsion.

23. The process according to any one of claims 1 to 11 or any one of claims 17 to 22 or the stored influenza vaccine according to any one of claims 12 to 22 substantially as hereinbefore described with reference to the accompanying Figures and/or Examples.

DATED this TENTH day of SEPTEMBER, 2013

Novartis Vaccines and Diagnostics GmbH
By patent attorneys for the applicant:
FB Rice

FIGURE 1

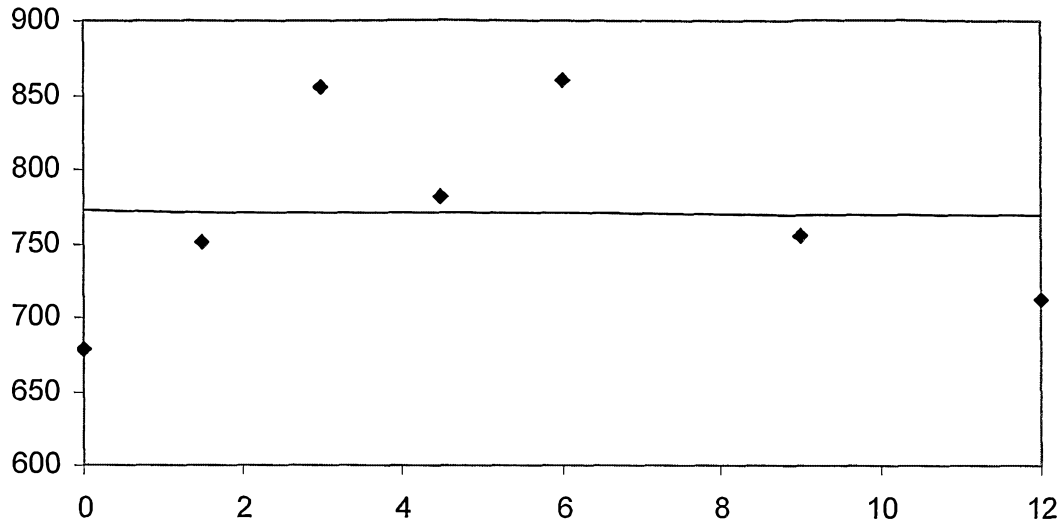

FIGURE 2

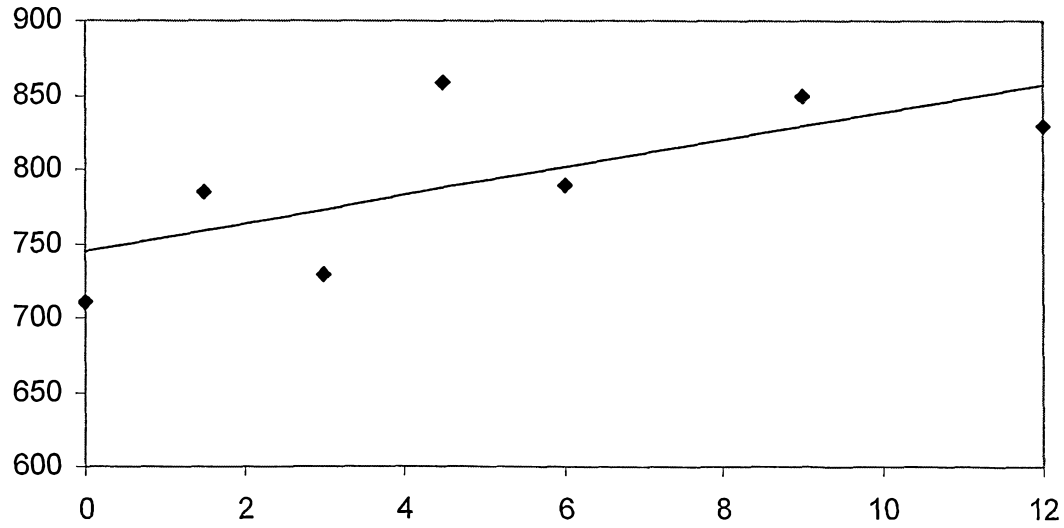

FIGURE 3

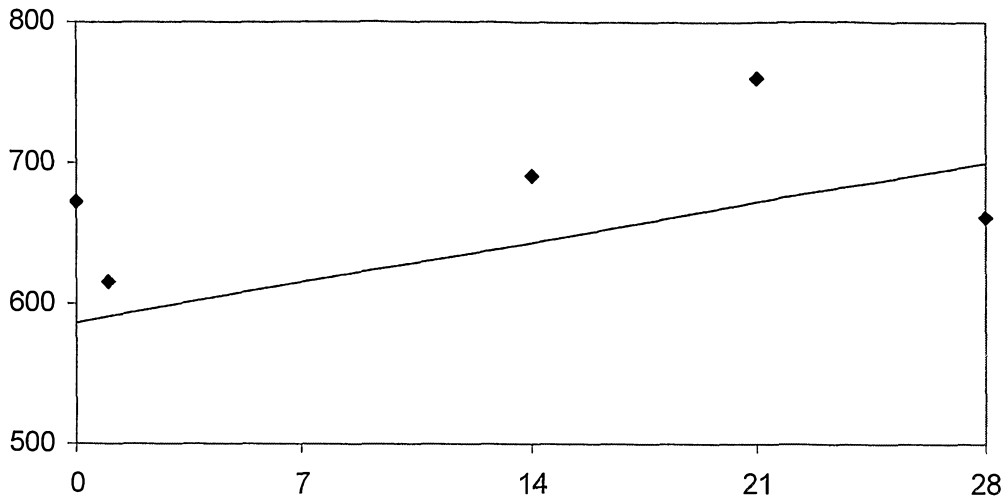

FIGURE 4

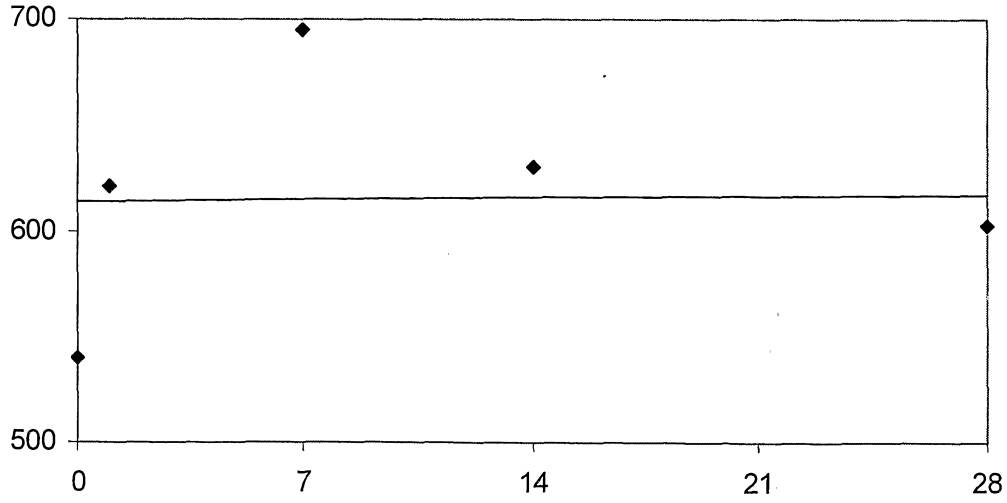

FIGURE 5

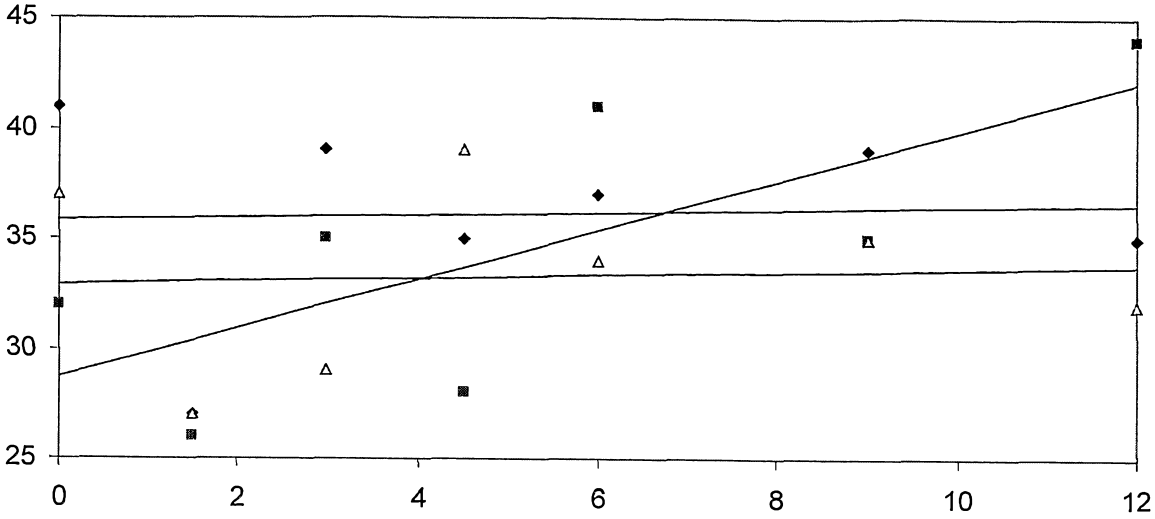

FIGURE 6

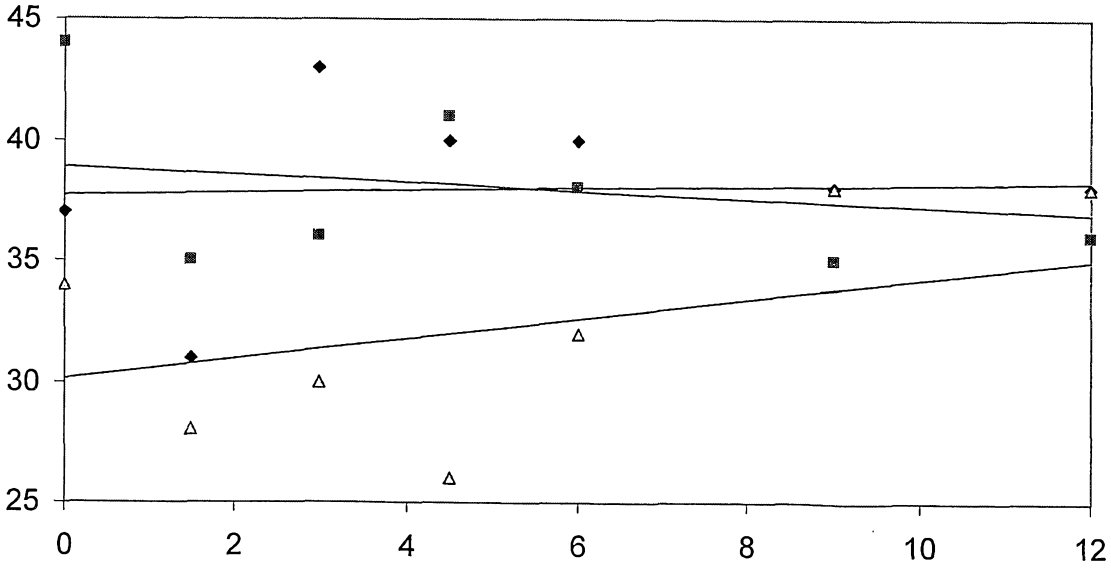

FIGURE 7

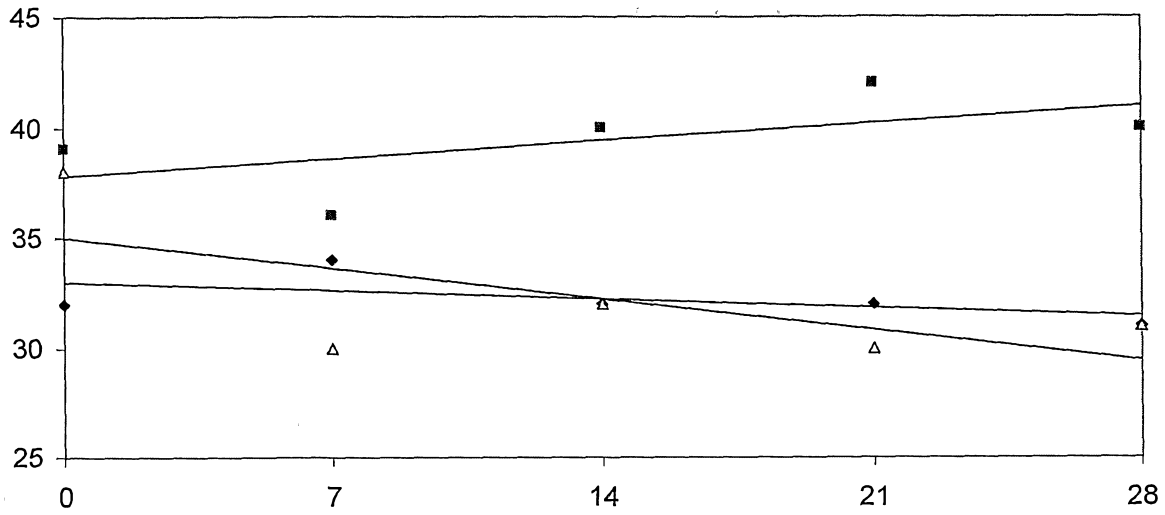

FIGURE 8