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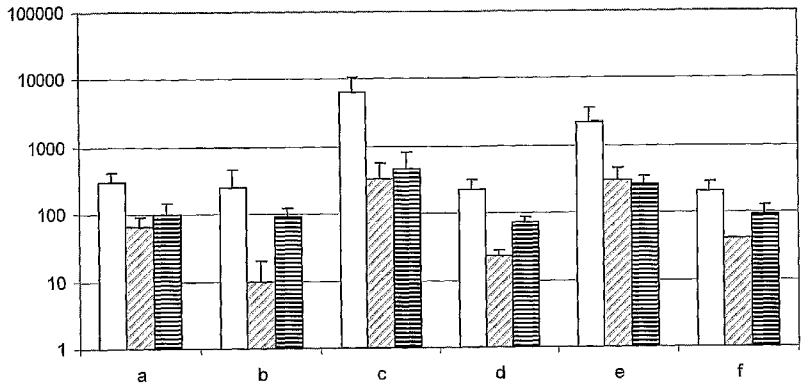
(54) **Title:** ADMINSTRATION ROUTES FOR PRIMING/BOOSTING WITH INFLUENZA VACCINES



(57) **Abstract:** Patients receive a mucosal influenza vaccine and then receive a parenteral influenza vaccine, in that order, typically during different visits to a vaccination centre.

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ADMINISTRATION ROUTES FOR PRIMING/BOOSTING WITH INFLUENZA VACCINES

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

TECHNICAL FIELD

This invention is in the field of the administration of influenza vaccines to patients.

BACKGROUND ART

Influenza vaccines currently in general use are described in more detail in chapters 17 & 18 of reference 1. They are based on live virus or inactivated virus, and inactivated vaccines can be based on whole virus, 'split' virus or on purified surface antigens (including haemagglutinin and neuraminidase). Haemagglutinin (HA) is the main immunogen in inactivated influenza vaccines, and vaccine doses are standardized by reference to HA levels, with vaccines typically containing about 15μg of HA per strain.

Most current vaccines are administered to patients parenterally, by intramuscular injection. The FLUMIST™ product, however, is a live attenuated vaccine that is administered intranasally, which gives access to the mucosal immune system. This nasal vaccine is administered by a dosage schedule where a first 0.5 mL dose is followed by a second 0.5 mL dose at least 6 weeks later.

Thus parenteral and mucosal routes are each currently used for administration of influenza vaccines.

It has also been proposed to administer vaccines to patients by both of these routes. For example, reference 58 discloses a two-dose regimen for influenza vaccination in which a patient receives a parenteral dose (typically intramuscularly) and a mucosal dose (typically intranasally). These two vaccines are preferably administered to a patient during a single visit to a physician. The inclusion of a mucosal dose in the two-dose regimen is said to enhance the protective immune response achieved by the vaccine, and in particular to enhance the IgA antibody response. Reference 2 discloses a three-dose regimen, with mice receiving two doses of an adjuvanted monovalent vaccine by subcutaneous injection, followed by an unadjuvanted booster by the intranasal route.

The natural infection route of the influenza virus is through the upper and lower respiratory tract. While the upper respiratory tract is mainly protected by locally derived IgA, the lower respiratory tract is mainly protected by serum or locally derived IgG, in both humans and animals. Thus methods that induce both IgA and IgG responses may provide better protection than methods that provide only one of these two responses.

It is an object of the invention to provide further and improved multi-dose regimens for administration of influenza vaccines. In particular, it is an object of the invention to provide such regimens such that IgA and IgG responses can be elicited.

DISCLOSURE OF THE INVENTION

According to the invention, patients receive a mucosal influenza vaccine and then receive a parenteral influenza vaccine. The two vaccines are given in this order *i.e.* mucosal first. The two

vaccines will generally not be given at substantially the same time *i.e.* they will not be administered during the same visit to a vaccination centre. Rather, they will be given at least 1 day apart from each other *e.g.* several weeks apart. Separation of dosing in this way has been found to give the best immune responses.

Thus the invention provides a process for immunizing a patient against influenza virus infection, wherein a first influenza vaccine is administered to the patient and then a second influenza vaccine is administered to the patient, wherein the first vaccine is administered by a mucosal route and the second vaccine is administered by a parenteral route. The mucosally-administered vaccine and the parenterally-administered vaccine will usually be antigenically the same as each other, but they may be antigenically different (see below). The mucosally-administered vaccine and the parenterally-administered vaccine will usually differ in terms of non-antigenic components *e.g.* they may include different carriers, delivery systems, adjuvants, *etc.*

The invention also provides the use of influenza antigens in the manufacture of a multi-dose vaccine for immunizing against influenza virus infection, wherein said multi-dose vaccine is administered to a patient by a treatment regimen in which a first influenza vaccine is administered to the patient and then a second influenza vaccine is administered to the patient, wherein the first vaccine is administered by a mucosal route and the second vaccine is administered by a parenteral route.

The invention also provides a process for administering a second influenza vaccine to a patient who has previously received a first influenza vaccine by a mucosal route, wherein said second vaccine is administered to the patient by a parenteral route.

The invention also provides the use of an influenza antigen in the manufacture of a vaccine for immunizing against influenza virus infection, wherein (i) the vaccine is for administration to a patient by a parenteral route, and (ii) the patient has previously received an influenza vaccine by a mucosal route.

The time between administration of the initial mucosal dose and subsequent administration of the parenteral dose is typically at least *n* days, where *n* is selected from 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 42, 49, 56 or more. The time will typically be no longer than 6 months. The doses may be given about 4 weeks apart from each other *e.g.* at day 0 and then at about day 28.

The preferred parenteral administration route is injection, typically intramuscular injection.

Preferred mucosal administration routes are oral and, more preferably, intranasal.

The mucosal vaccine and/or the parenteral vaccine may be adjuvanted. As an alternative, either or both of them may be adjuvanted. Where both are adjuvanted, they may use the same adjuvant or, more typically, they will use different adjuvants.

The form of influenza antigen in current vaccines is either live virus or inactivated virus, and the antigen in inactivated vaccines can take the form of whole virus, 'split' virus or purified surface

antigens. The mucosal vaccine and/or the parenteral vaccine can use different forms of antigen, but they will typically both use the same form of antigen.

The invention also provides a kit comprising: (i) a first influenza vaccine packaged for administration to a patient by a mucosal route; and (ii) a second influenza vaccine packaged for administration to a patient by a parenteral route. The kit may also include instructions to administer the first vaccine by a mucosal route and the second vaccine by a parenteral route.

The influenza virus antigen

The invention involves the use of two separate influenza vaccines: a first mucosal vaccine and a second parenteral vaccine. Each of these two vaccines will include an influenza virus antigen. The antigen in each vaccine will typically be prepared from influenza virions but, as an alternative, antigens such as haemagglutinin can be expressed in a recombinant host (*e.g.* in yeast using a plasmid expression system, or in an insect cell line using a baculovirus vector) and used in purified form [3,4]. In general, however, antigens will be from virions.

The antigen may take the form of a live virus or 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, 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.

Where an inactivated virus is used, the vaccine may comprise whole virion, split virion, or purified surface antigens (including hemagglutinin and, usually, also including neuraminidase).

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 virions with detergents (*e.g.* ethyl ether, polysorbate 80, deoxycholate, tri-*N*-butyl phosphate, Triton X-100, Triton N101, cetyltrimethylammonium bromide, *etc.*) to produce subvirion preparations, including the 'Tween-ether' splitting process. Methods of splitting influenza viruses are well known in the art *e.g.* see refs. 5-10, *etc.* Splitting of 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 DOT-MA, 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 [11] (nucleic acid free viral-like liposomal particles), as in the INFLEXAL V™ and INVAVAC™ products. Virus-like particles (VLPs) may also be used.

The influenza virus may be attenuated. The influenza virus may be temperature-sensitive. The influenza virus may be cold-adapted. These three possibilities apply in particular for live viruses.

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 viruses 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 influenza A virus HA subtypes H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15 or H16. The invention may protect against one or more of influenza A virus NA subtypes N1, N2, N3, N4, N5, N6, N7, N8 or N9.

Other strains that can usefully be included in the compositions are strains which are resistant to antiviral therapy (*e.g.* resistant to oseltamivir [12] and/or zanamivir), including resistant pandemic strains [13].

The adjuvanted 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 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), such that the human population will

be immunologically naïve to the strain's hemagglutinin; (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 immunising 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 [14], with clades 1 and 3 being particularly relevant.

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. 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. For the two vaccines used according to the invention, it is preferred that they will have at least one viral strain in common, and it is more preferred that the strain(s) in both vaccines are identical.

The influenza virus may be a reassortant strain, and may have been obtained by reverse genetics techniques. Reverse genetics techniques [*e.g.* 15-19] allow influenza viruses with desired genome segments to be prepared *in vitro* using plasmids. Typically, it involves expressing (a) DNA molecules that encode desired viral RNA molecules *e.g.* from 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 [20-22], and these methods will also involve the use of plasmids to express all or some (*e.g.* just the PB1, PB2, PA and NP proteins) of the viral proteins, with 12 plasmids being used in some methods.

To reduce the number of plasmids needed, a recent approach [23] combines a plurality of RNA polymerase I transcription cassettes (for viral RNA synthesis) on the same plasmid (*e.g.* sequences encoding 1, 2, 3, 4, 5, 6, 7 or all 8 influenza A vRNA segments), and a plurality of protein-coding regions with RNA polymerase II promoters on another plasmid (*e.g.* sequences encoding 1, 2, 3, 4, 5, 6, 7 or all 8 influenza A mRNA transcripts). Preferred aspects of the reference 23 method involve: (a) PB1, PB2 and PA mRNA-encoding regions on a single plasmid; and (b) all 8 vRNA-encoding segments on a single plasmid. Including the NA and HA segments on one plasmid and the six other segments on another plasmid can also facilitate matters.

As an alternative to using polI promoters to encode the viral RNA segments, it is possible to use bacteriophage polymerase promoters [24]. For instance, promoters for the SP6, T3 or T7 polymerases can conveniently be used. Because of the species-specificity of polI promoters, bacteriophage polymerase promoters can be more convenient for many cell types (*e.g.* MDCK), although a cell must also be transfected with a plasmid encoding the exogenous polymerase enzyme.

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 [25,26].

Thus 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), particularly when viruses are grown in eggs. 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.

The viruses used as the source of the antigens can be grown either on eggs or on cell culture. 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). More recently, however, viruses have been grown in animal cell culture and, for reasons of speed and patient allergies, this growth method is preferred. 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 [10].

When cell culture is used, the viral growth 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.*. Preferred mammalian cell lines for growing influenza viruses include: MDCK cells [27-30], derived from Madin Darby canine kidney; Vero cells [31-33], derived from African green monkey (*Cercopithecus aethiops*) kidney; or PER.C6 cells [34], derived from human embryonic retinoblasts. These cell lines are widely available *e.g.* from the American Type Cell Culture (ATCC) collection [35], from the Coriell Cell Repositories [36], or from the European Collection of Cell Cultures (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 a less-preferred alternative to mammalian cell lines, virus can be grown on avian cell lines [*e.g.* refs. 37-39], including avian embryonic stem cells [37,40] and cell lines derived from ducks (*e.g.* duck retina), or from hens. Suitable avian embryonic stem cells, include the EBx cell line derived from chicken embryonic stem cells, EB45, EB14, and EB14-074 [41]. Chicken embryo fibroblasts (CEF), can also be used, *etc.*

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

Where virus has been grown on a mammalian cell line then the composition will advantageously be free from egg proteins (*e.g.* ovalbumin and ovomucoid) and from chicken DNA, thereby reducing allergenicity.

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

Where virus has been grown on a cell line then 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 [46,47]. 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 [48]; immunoassay methods, such as the Threshold™ System [49]; and quantitative PCR [50]. 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 [49]. 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 51.

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 52 & 53, 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 [54].

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.

It is preferred that the average length of any residual host cell DNA is less than 500bp *e.g.* less than 400bp, less than 300bp, less than 200bp, less than 100bp, *etc.*

For growth on a cell line, such as on MDCK cells, virus may be grown on cells in suspension [27,55,56] or in adherent culture. One suitable MDCK cell line for 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 [57] (*e.g.* 30-36°C, or at about 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C), for example 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.

Haemagglutinin (HA) is the main immunogen in inactivated influenza vaccines, and vaccine doses are standardised by reference to HA levels, typically as measured by a single radial immunodiffusion (SRID) assay. Vaccines typically contain about 15μg of HA per strain, although lower doses are also used *e.g.* for children, or in pandemic situations. Fractional doses such as ½ (*i.e.* 7.5μg HA per strain), ¼ and $^1/_8$ have been used [58,59], as have higher doses (*e.g.* 3x or 9x doses [60,61]). 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 45, about 30, about 15, about 10, about 7.5, about 5, about 3.8, about 1.9, about 1.5, *etc.* per strain. These lower doses are most useful when an adjuvant is present in the vaccine, as with the invention. 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.

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 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.

Compositions of the invention may include 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. The detergent may be present only at trace amounts. Thus the vaccine may included less than 1mg/ml of each of octoxynol-10,

α-tocopheryl hydrogen succinate and polysorbate 80. Other residual components in trace amounts could be antibiotics (*e.g.* neomycin, kanamycin, polymyxin B).

An inactivated but non-whole cell vaccine (*e.g.* a split virus vaccine or a purified surface antigen vaccine) may include matrix protein, in order to benefit from the additional T cell epitopes that are located within this antigen. Thus a non-whole cell vaccine (particularly a split vaccine) that includes haemagglutinin and neuraminidase may additionally include M1 and/or M2 matrix protein. Where a matrix protein is present, inclusion of detectable levels of M1 matrix protein is preferred. Nucleoprotein may also be present.

Pharmaceutical compositions

Vaccines used with the invention are pharmaceutically acceptable. They may include components in addition to the antigen and adjuvant *e.g.* they will typically include one or more pharmaceutical carrier(s) and/or excipient(s). A thorough discussion of such components is available in reference 62. The carrier(s)/excipient(s) used in mucosal vaccines may be the same as or different from those used in parenteral vaccines.

Compositions may include preservatives such as thiomersal or 2-phenoxyethanol. It is preferred, however, that the vaccines should be substantially free from (*i.e.* less than 5μg/ml) mercurial material *e.g.* thiomersal-free [9,63]. Vaccines containing no mercury are more preferred.

To control tonicity, particularly in injectable vaccines, 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 for injection 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 [64], 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 pH of a composition will generally be between 5.0 and 8.1, and more typically between 6.0 and 8.0 *e.g.* between 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 composition may include material for a single immunisation, or may include material for multiple immunisations (*i.e.* a 'multidose' kit). 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. For intranasal administration, this total dosage volume can be split between nostrils *e.g.* ½ in each nostril.

Compositions and kits are preferably stored at between 2°C and 8°C. They should not be frozen. They should ideally be kept out of direct light.

Packaging of compositions or kit components

Suitable containers for compositions of the invention (or kit components) include vials, syringes (*e.g.* disposable syringes), nasal sprays, *etc*. These containers 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 (*e.g.* to reconstitute lyophilised material therein), 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 will not normally have a needle attached to it, although a separate needle may be supplied with the syringe for assembly and use. 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 kit or composition may be packaged (*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

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) [65]. 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.

For mucosal administration of vaccines, routes that may be used include, but are not limited to, rectal, oral (*e.g.* tablet, spray), pharyngeal, buccal, vaginal, topical, transdermal or transcutaneous, intranasal, ocular, pulmonary, *etc.* As mentioned above, the preferred mucosal administration route is by intranasal injection. Nasal administration can be *e.g.* by spray, drops, aerosol, *etc.*

For parenteral administration of vaccines, routes that may be used include, but are not limited to, intramuscular injection, subcutaneous injection, intravenous injection, intraperitoneal injection (where available), intradermal injection, *etc*, and other systemic routes. As mentioned above, the preferred parenteral administration route is by intramuscular injection (*e.g.* into the arm or leg).

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, 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.

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™).

Adjuvant(s)

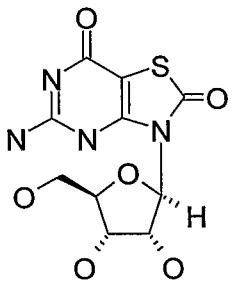
The mucosal vaccine and/or the parenteral vaccine may be unadjuvanted, or they may be administered with an adjuvant. The adjuvant(s) can function to enhance the immune responses (humoral and/or cellular) elicited in a patient who receives the composition. Some adjuvants are effective for parenteral administration but not for mucosal administration (*e.g.* aluminum salts), and *vice versa*, although some adjuvants are effective for both routes. Where adjuvants are used, they will be chosen accordingly.

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

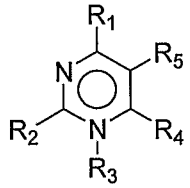
- A mineral-containing composition, including calcium salts and aluminum salts (or mixtures thereof). Calcium salts include calcium phosphate (*e.g.* the "CAP" particles disclosed in ref.

66). 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 [67]. Aluminum salt adjuvants are described in more detail below.

- An oil-in-water emulsion, as described in more detail below.
- An immunostimulatory oligonucleotide, as described in more detail below.
- 3-O-deacylated monophosphoryl lipid A ('3dMPL', also known as 'MPL™'), as described in more detail below.
- An imidazoquinoline compound, such as Imiquimod ("R-837") [68,69], Resiquimod ("R-848") [70], and their analogs; and salts thereof (*e.g.* the hydrochloride salts). Further details about immunostimulatory imidazoquinolines can be found in references 71 to 75.
- A thiosemicarbazone compound, such as those disclosed in reference 76. Methods of formulating, manufacturing, and screening for active compounds are also described in reference 76. 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 77. Methods of formulating, manufacturing, and screening for active compounds are also described in reference 77. 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 78 to 80; (f) a compound having the formula:

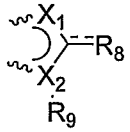


wherein:

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}:



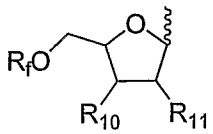
R_{4-5}

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) [81].

- Compounds disclosed in reference 82, including: Acylpiperazine compounds, Indoledione compounds, Tetrahydraisoquinoline (THIQ) compounds, Benzocyclodione compounds, Aminoazavinyl compounds, Aminobenzimidazole quinolinone (ABIQ) compounds [83,84], Hydrapthalamide compounds, Benzophenone compounds, Isoxazole compounds, Sterol compounds, Quinazilinone compounds, Pyrrole compounds [85], Anthraquinone compounds, Quinoxaline compounds, Triazine compounds, Pyrazalopyrimidine compounds, and Benzazole compounds [86].
- A polyoxidonium polymer [87,88] or other N-oxidized polyethylene-piperazine derivative.
- Compounds disclosed in reference 89, including 3,4-di(1H-indol-3-yl)-1H-pyrrole-2,5-diones, staurosporine analogs, derivatized pyridazines, chromen-4-ones, indolinones, quinazolines, and nucleoside analogs.
- An aminoalkyl glucosaminide phosphate derivative, such as RC-529 [90,91].
- A CD1d ligand, such as an α-glycosylceramide [92-99] (*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 100 and 101.
- 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

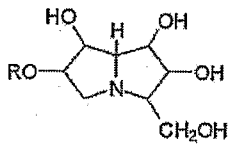
1-[4-amino-2-(propylamino)-1H-imidazo[4,5-c]quinolin-1-yl]-2-methylpropan-2-ol

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

- Saponins [chapter 22 of ref. 129], 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. 102. Saponin formulations may also comprise a sterol, such as cholesterol [103]. Combinations of saponins and cholesterols can be used to form unique particles called immunostimulating complexs (ISCOMs) [chapter 23 of ref. 129]. 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. 103-105. Optionally, the ISCOMS may be devoid of additional detergent [106]. A review of the development of saponin based adjuvants can be found in refs. 107 & 108.
- 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 [109]. The use of detoxified ADP-ribosylating toxins as mucosal adjuvants is described in ref. 110 and as parenteral adjuvants in ref. 111.
- Bioadhesives and mucoadhesives, such as esterified hyaluronic acid microspheres [112] or chitosan and its derivatives [113].
- 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).

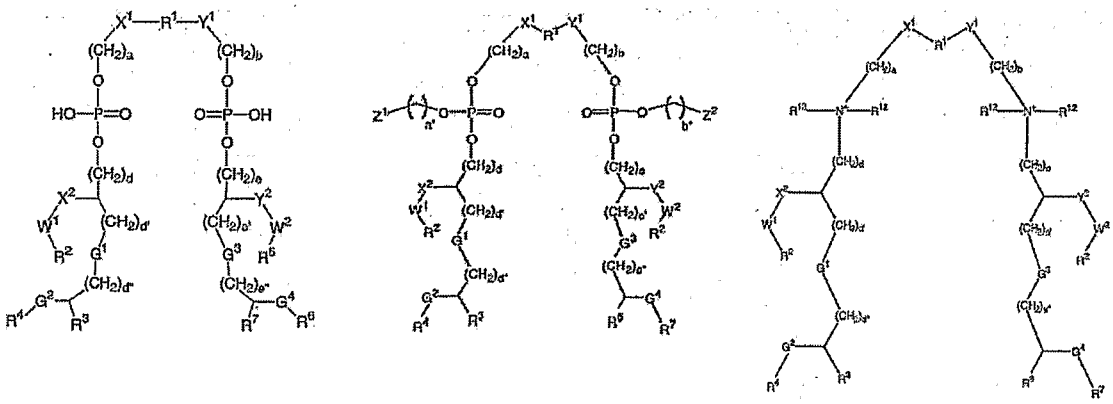
- Liposomes (Chapters 13 & 14 of ref. 129). Examples of liposome formulations suitable for use as adjuvants are described in refs. 114-116.
- Polyoxyethylene ethers and polyoxyethylene esters [117]. Such formulations further include polyoxyethylene sorbitan ester surfactants in combination with an octoxynol [118] as well as polyoxyethylene alkyl ethers or ester surfactants in combination with at least one additional non-ionic surfactant such as an octoxynol [119]. 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 (LPS) preparation derived from a second Gram-negative bacterium, wherein the outer membrane protein proteosome and LPS preparations form a stable non-covalent adjuvant complex. Such complexes include "IVX-908", a complex comprised of *Neisseria meningitidis* outer membrane and LPS.
- Methyl inosine 5'-monophosphate ("MIMP") [120].
- A polyhydroxlated pyrrolizidine compound [121], such as one having formula:



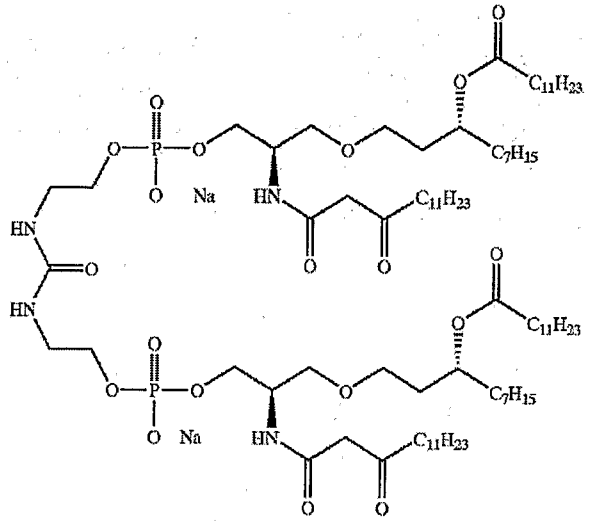
where R is selected from the group comprising hydrogen, straight or branched, unsubstituted or substituted, saturated or unsaturated acyl, alkyl (*e.g.* cycloalkyl), alkenyl, alkynyl and aryl groups, or a pharmaceutically acceptable salt or derivative thereof. Examples include, but are not limited to: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

- A gamma inulin [122] or derivative thereof, such as algammulin.
- A compound of formula I, II or III, or a salt thereof:

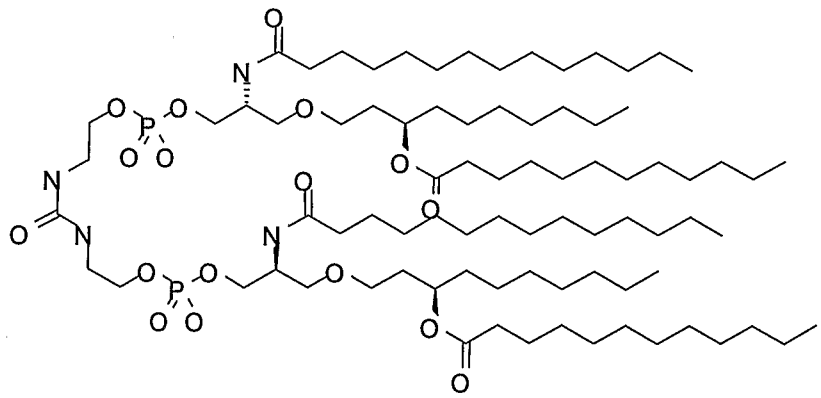
I II III



as defined in reference 123, 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

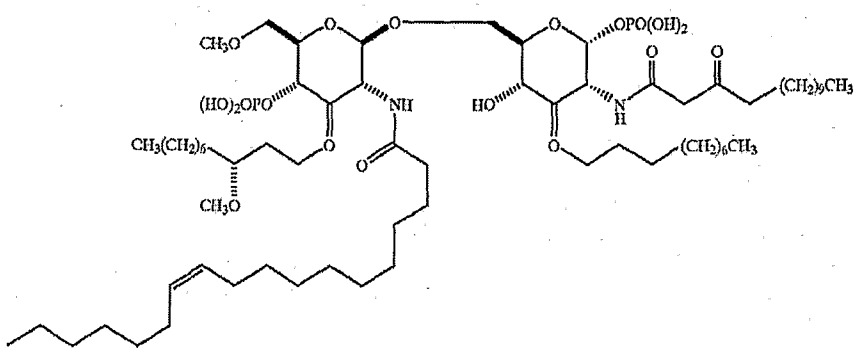


ER-803022:

- Derivatives of lipid A from *Escherichia coli* such as OM-174 (described in refs. 124 & 125).
- 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 [126].

- Compounds containing lipids linked to a phosphate-containing acyclic backbone, such as the TLR4 antagonist E5564 [127,128]:



These and other adjuvant-active substances are discussed in more detail in references 129 & 133.

The adjuvant(s) 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.

A single vaccine may include two or more of said adjuvants.

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

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 129). 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. 129]. 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. 129].

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. 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.

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. Non-ionic surfactants are preferred. Preferred surfactants for including in the emulsion are Tween 80 (polyoxyethylene sorbitan monooleate), Span 85 (sorbitan trioleate), lecithin and Triton X-100.

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' [130-132], as described in more detail in Chapter 10 of ref. and chapter 12 of ref. 133. 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 [134] (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 [135] (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 136, 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 137, 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 in which a saponin (*e.g.* QuilA or QS21) and a sterol (*e.g.* a cholesterol) are associated as helical micelles [138].
- 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) [139].
- 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) [139].

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 [140]. They also have antioxidant properties that may help to stabilize the emulsions [141]. 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 [9]. Preservative-free vaccines are particularly preferred.

Immunostimulatory oligonucleotides

Immunostimulatory oligonucleotides can include nucleotide modifications/analogs such as phosphorothioate modifications and can be double-stranded or (except for RNA) single-stranded. References 142, 143 and 144 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. 145-150. A CpG sequence may be directed to TLR9, such as the motif GTCGTT or TTCGTT [151]. 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. 152-154. 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 151 & 155-157. 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 [158]. 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. 158), 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. 158), 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.

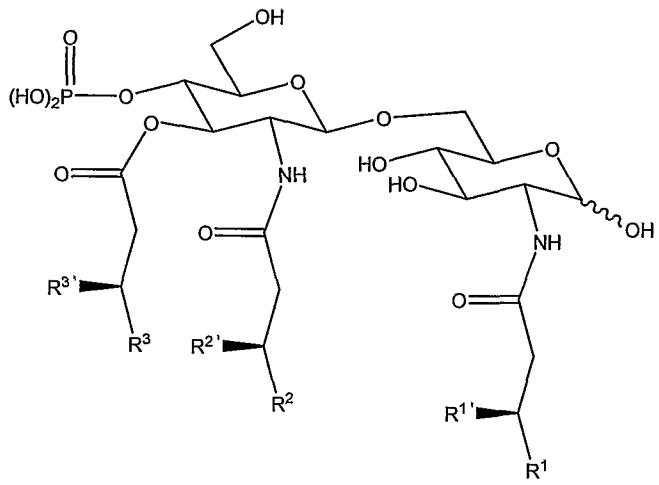
Immunostimulatory oligonucleotides will typically comprise at least 20 nucleotides. They may comprise fewer than 100 nucleotides.

3 de-O-acylated monophosphoryl lipid A

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. 159). Preparation of 3dMPL was originally described in reference 160.

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:

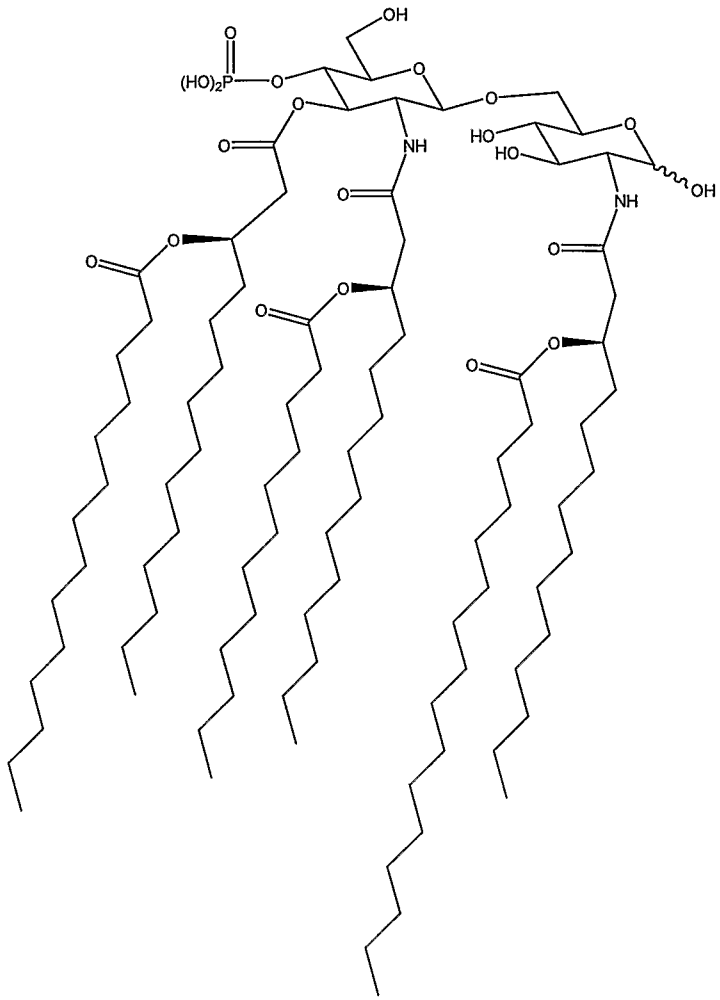


Groups R^1, R^2 and R^3 are each independently $-(CH_2)_n-CH_3$. The value of *n* is preferably between 8 and 16, more preferably between 9 and 12, and is most preferably 10.

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 [161]. 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*±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.

A typical amount of 3dMPL in a vaccine is 10-100μg/dose *e.g.* about 25μg or about 50μg.

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 [162] (including in an oil-in-water emulsion [163]), with an immunostimulatory oligonucleotide, with both QS21 and an immunostimulatory oligonucleotide, with aluminum phosphate [164], with aluminum hydroxide [165], or with both aluminum phosphate and aluminum hydroxide.

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.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 shows serum HI titers in mice immunised by regimens (a) to (f). In each of the six groups, the three bars are, from left to right: A/Wyoming; A/New Caledonia; B/Jiangsu.

Figure 2 shows CLN ELISPOT results, showing antibody secreting cells per million mononuclear cells, for regimens (a) to (e). White bars are IgA, grey bars are IgG.

Figure 3 shows nasal wash IgA titers for regimens (a) to (e).

Figures 4 and 5 show cytokine levels (pg/ml) in cervical lymph nodes (Figure 4) or spleen (Figure 5), using regimens (a) to (d). For each of the four regimens, the three bars are, from left to right: IFNγ; IL-13; and IL-5.

MODES FOR CARRYING OUT THE INVENTION

Trivalent influenza subunit vaccines were prepared from viruses grown on MDCK cell culture. The strains were: (i) A/Wyoming H3N2; (ii) A/New Caledonia H1N1; and (iii) B/Jiangsu. These vaccines were used to immunize female BALB/c mice by a variety of 2-dose regimens, with doses being given at day 0 and day 28: (a) 2 x intramuscular injection; (b) 2 x intranasal spray; (c) intranasal spray then intramuscular injection; (d) intramuscular injection then intranasal spray; (e) 2 x simultaneous intramuscular injection and intranasal spray. A sixth group (f) received a single instance of simultaneous intramuscular injection and intranasal spray. The intranasal formulations included the LT-K63 adjuvant at 5μg. The HA dose per strain per vaccine dose was 1μg. Serum samples, nasal washes and bronchoalveolar lavage (BAL) were taken at day 42 and assayed for serum IgG (ELISA), mucosal IgA (ELISA) and haemagglutination inhibition.

Overall, regimen (c) consistently induced the highest serum HI titers *i.e.* an intranasal administration followed by an intramuscular injection. This regimen induced ≥10-fold higher HI titers than all other routes of immunizations (Figure 1) and reached statistical significance compared to all other groups ($p<0.002$, student's t test (two tail, two sample assuming equal variances), 95% confidence interval).

In contrast, reversal of the two doses, *i.e.* regimen (d), gave the poorest response. The next poorest were (b) then (a). Thus a regimen involving separate mucosal and parenteral doses, with the mucosal dose being administered first, seems optimal. Separating the two doses, rather than giving them on the same day, gives a more potent response.

In further experiments where intramuscular injections included 1/10 of the HA dose (0.1μg HA per strain), the same pattern of serum HI titers was seen. The pattern was changed, however, if the antigen doses were maintained (1μg HA per strain in both the intramuscular and intranasal doses) while the amount of LT-K63 in the intranasal dose was reduced 10-fold (0.5μg). While regimen (c) was still superior to regimen (d), regimen (e) gave the best overall titers. In contrast, regimen (c) gave the best results if these 10-fold reductions were both used *i.e.* 0.1μg HA in the intramuscular injection and 0.5μg LT-K63 in the intranasal doses. Where an adjuvant is used, therefore, the quantity should be sufficient for the amount of antigen also being administered.

As shown in Figure 2, anti-HA IgG and IgA antibody secreting cells were detected locally in cervical lymph nodes (CLN) after regimen (b). Regimens (c) and (d) induced only IgG antibody secreting cells in CLN. As expected, regimen (a) did not induce any IgG or IgA antibody secreting cells in CLN. The ELISPOT results were confirmed by ELISA on supernatants from overnight stimulations of CLN cells with HA.

Importantly, there was a correlation between antibody responses in the mucosal effector site (indirectly measured as IgA in nasal washes) and mucosal inductive site (CLN), because anti-HA IgA responses in nasal washes were highest in regimen (b), as seen in Figure 3. Regimen (c) induced the second highest anti-HA IgA responses in nasal washes, significantly higher ($p<0.02$) than regimen (a), followed by regimen (d). These data demonstrate that an initial mucosal dose, as in

regimens (b) and (c), induced the highest levels of anti-HA IgG and IgA local (CLN) and mucosal (nasal wash) responses. Thus a mucosal immunization followed by a parenteral immunization is useful for inducing local IgA with its many effector functions.

To determine whether the different regimens also resulted in increased cytokine responses, we also measured IFNγ, IL-13 and IL-5 in culture supernatants of CLN and spleens a week after the final immunization. Regimen (c) induced the highest amount of IFNγ, IL-13 and IL-5 locally in CLN as well as systemically in spleen (Figures 4 & 5). Notably, the cytokine responses were generally higher in CLN (Figure 4) compared to Spleen (Figure 5). These data again demonstrate the advantages of regimen (c).

The role of cytokines in immunity against influenza is well studied in animal models, and the data herein suggest that a balanced TH1 and TH2 response can be achieved, which may prove less pathological than an exclusive TH1 or TH2 type response.

Induction of mucosal immunity by inactivated poliovirus vaccine through parenteral immunization is dependent on previous mucosal contact with live virus [166]. Also, influenza-primed children exhibited significantly higher IgG and IgA responses than unprimed children [167]. Our data suggest that induction of mucosal and systemic responses following parenteral immunizations may be due to prior mucosal priming by cross-reacting virus strains. Thus regimen (c) may prove particularly effective at inducing pre-existing immunity against new influenza strains in a naïve population.

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

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CLAIMS

1. A process for immunizing a patient against influenza virus infection, wherein a first influenza vaccine is administered to the patient and then a second influenza vaccine is administered to the patient, wherein the first vaccine is administered by a mucosal route and the second vaccine is administered by a parenteral route.

2. The process of claim 1, wherein the time between administration of the mucosal dose and the parenteral dose at least 14 days.

3. The process of claim 1 or claim 2, wherein the time between administration of the mucosal dose and the parenteral dose is no longer than 6 months.

4. A process for administering a second influenza vaccine to a patient who has previously received a first influenza vaccine by a mucosal route, wherein said second vaccine is administered to the patient by a parenteral route.

5. The process of claim 4, wherein the previous mucosal influenza vaccine was received at least 14 days before the parenteral administration.

6. The process of claim 4 or claim 5, wherein the previous mucosal influenza vaccine was received no more than 6 months before the parenteral administration.

7. A kit comprising: (i) a first influenza vaccine packaged for administration to a patient by a mucosal route; and (ii) a second influenza vaccine packaged for administration to a patient by a parenteral route. The kit may also include instructions to administer the first vaccine by a mucosal route and the second vaccine by a parenteral route.

8. The kit or process of any preceding claim, wherein the parenteral administration route is intramuscular injection.

9. The kit or process of any preceding claim, wherein the mucosal administration route is intranasal.

10. The kit or process of any preceding claim, wherein the mucosal vaccine is adjuvanted.

11. The kit or process of any preceding claim, wherein the parenteral vaccine is adjuvanted.

12. The kit or process of any preceding claim, wherein the mucosal and parenteral vaccine are both adjuvanted.

13. The kit or process of any one of claims 1 to 10, wherein the parenteral vaccine is unadjuvanted.

14. The kit or process of any one of claims 10 to 13, wherein the mucosal vaccine is adjuvanted with a detoxified form of a bacterial ADP-ribosylating toxin.

15. The kit or process of any preceding claim, wherein the mucosal vaccine contains a live attenuated influenza virus.

16. The kit or process of any preceding claim, wherein the mucosal vaccine is live or inactivated.

17. The kit or process of any preceding claim, wherein the mucosal vaccine contains a live attenuated influenza virus.

18. The kit or process of any preceding claim, wherein the parenteral vaccine is an inactivated virus.

19. The kit or process of any preceding claim, wherein the parenteral vaccine is a split influenza vaccine for injection.

20. The kit or process of any one of claims 1 to 18, wherein the parenteral vaccine is a purified surface antigen influenza vaccine for injection.

21. The kit or process of any preceding claim, wherein the mucosal vaccine is prepared from viruses grown on cell culture.

22. The kit or process of any preceding claim, wherein the parenteral vaccine is prepared from viruses grown on cell culture.

23. The kit or process of any preceding claim, wherein the mucosal and parenteral vaccine are both prepared from viruses grown on cell culture.

24. Use of influenza antigens in the manufacture of a multi-dose vaccine for immunizing against influenza virus infection, wherein said multi dose vaccine is administered to a patient by a treatment regimen in which a first influenza vaccine is administered to the patient and then a second influenza vaccine is administered to the patient, wherein the first vaccine is administered by a mucosal route and the second vaccine is administered by a parenteral route.

FIGURE 1

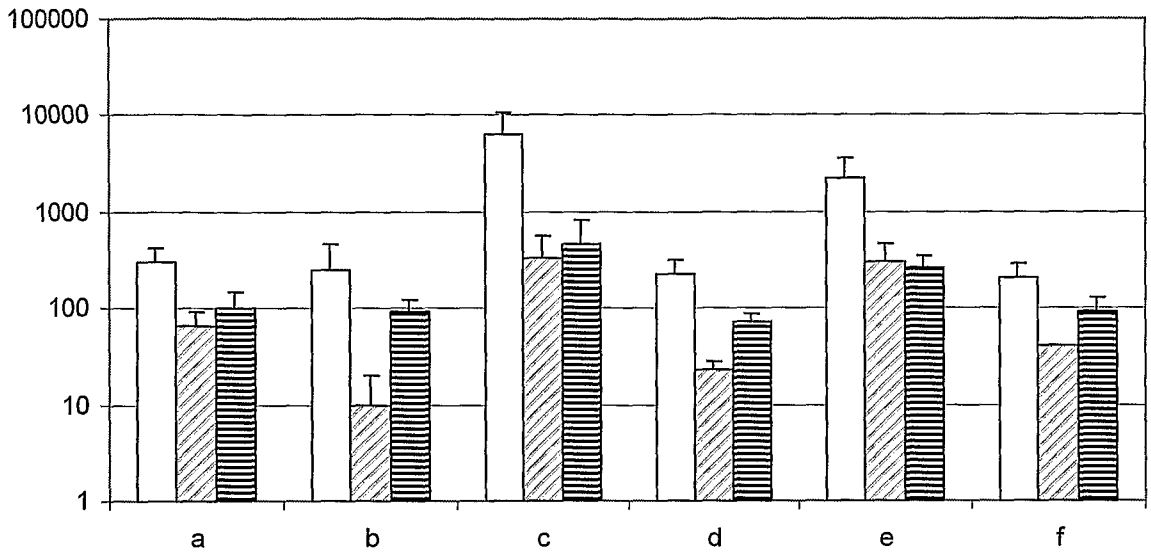

FIGURE 2

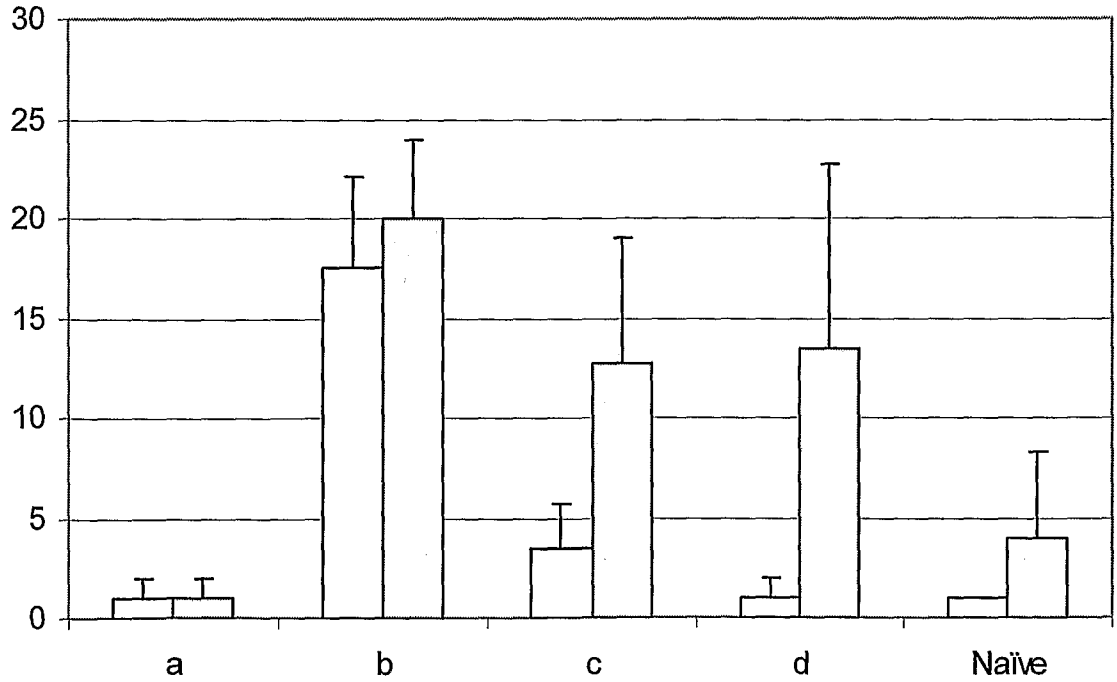

FIGURE 3

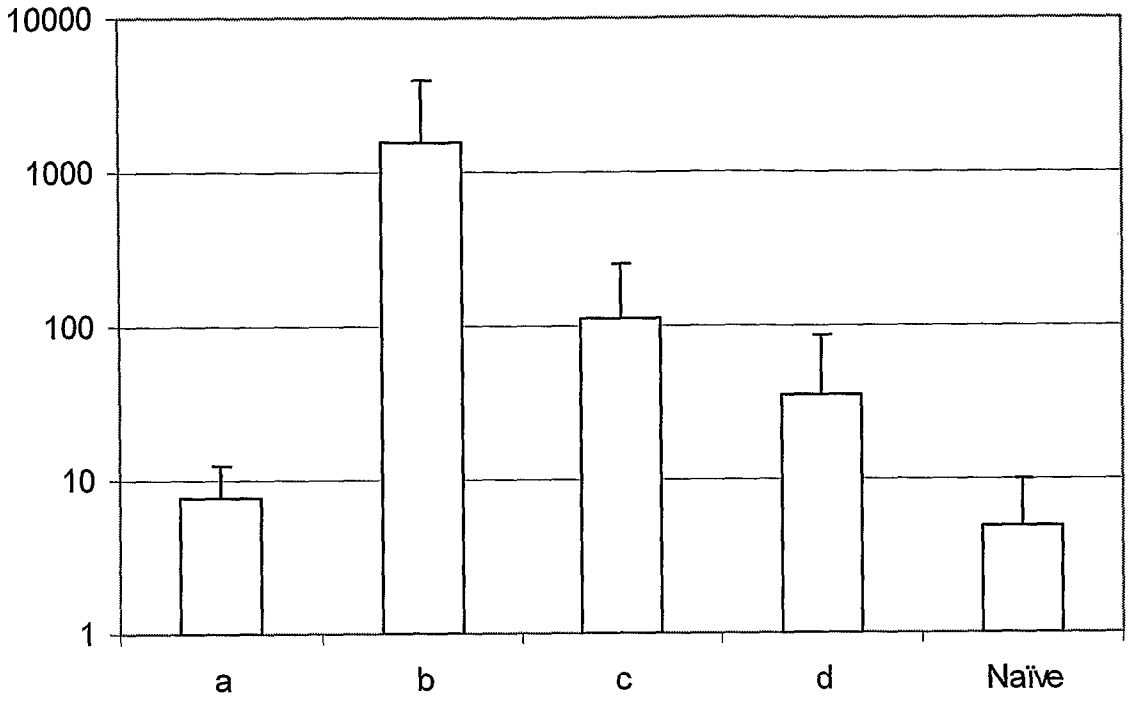

FIGURE 4

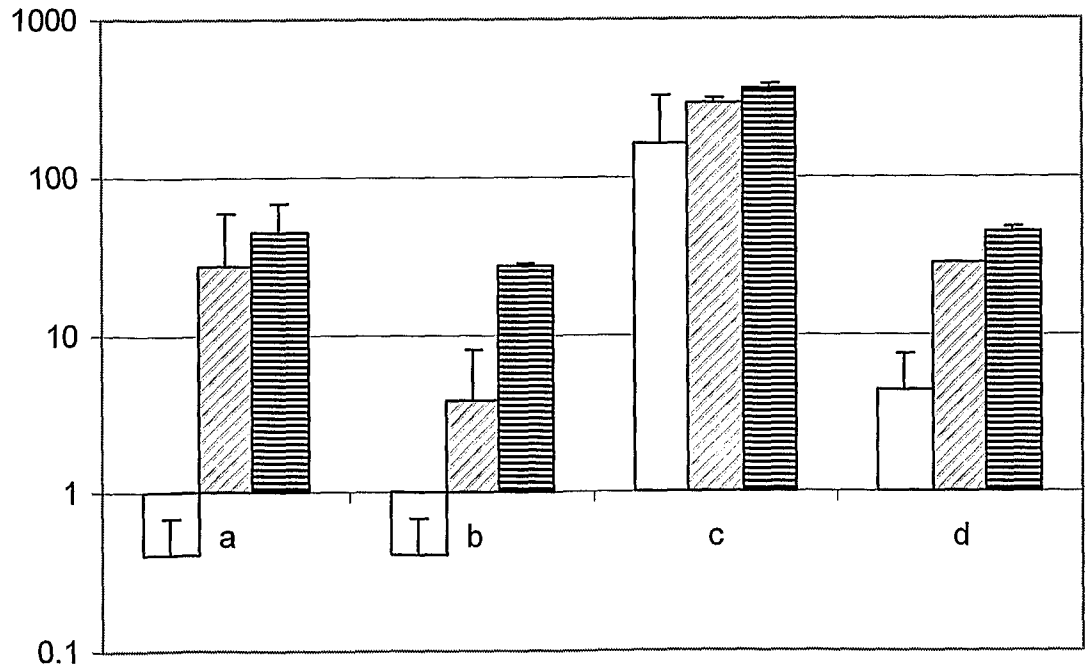

FIGURE 5