VACCINE

TECHNICAL FIELD

The present invention relates to improved vaccine and immunogenic compositions and their use in medicine. In particular the invention relates to vaccine or immunogenic formulations comprising an oil-in-water emulsion adjuvant and their use in medicine, in particular their use in augmenting immune responses to various antigens including *S. pneumoniae* antigens, and to methods of preparation, wherein the oil in water emulsion comprises a tocol, a metabolisable oil and an emulsifying agent.

TECHNICAL BACKGROUND

New compositions or vaccines with an improved immunogenicity are always needed. As one strategy, adjuvants have been used to try and improve the immune response raised to any given antigen and/or reduce reactogenicity/toxicity in the host.

Oil in water emulsions *per se* are well known in the art, and have been suggested to be useful as adjuvant compositions (EP 399843; WO 95/17210).

WO95/17210 discloses oil in water emulsions comprising from 2 to 10% squalene, from 2 to 10% alpha tocopherol and from 0.3 to 3% tween 80 and their use alone or in combination with QS21 and/or 3D-MPL.

WO99/12565 discloses oil in water emulsion compositions comprising a metabolisable oil, a saponin and a sterol. The oil in water emulsions further comprise 3D-MPL.

WO99/11241 discloses oil in water emulsions comprising metabolisable oil and a saponin, wherein the oil and saponin are present in a ratio of between 1:1 and 200:1.

There is still a need for improved vaccine and immunogenic compositions that provide a suitable immune response and are less reactogenic in the host.

STATEMENT OF THE INVENTION

The present inventors have discovered vaccine or immunogenic compositions comprising lower amounts of each component of the oil in water emulsion may be used whilst still

maintaining a comparable immune response against an antigen or antigenic composition within said composition. This carries the advantage maintaining the level of immunogenicity against an antigen whilst the reactogenicity within the host recipient is reduced.

Accordingly, in the first aspect of the present invention there is provided an immunogenic composition comprising a *Streptococcus pneumoniae* antigen, and an adjuvant composition comprising an oil-in-water emulsion, wherein said oil-in-water emulsion comprises 0.5 - 10 mg metabolisable oil, 0.5 – 11 mg tocol and 0.4 – 4 mg emulsifying agent, per human dose.

In another aspect of the present invention, there is provided a vaccine composition comprising a *Streptococcus pneumoniae* antigen, and an adjuvant composition comprising an oil-in-water emulsion, wherein said oil-in-water emulsion comprises 0.5 - 10 mg metabolisable oil, 0.5 – 11 mg tocol and 0.4 – 4 mg emulsifying agent, per human dose.

In a further aspect of the invention there is provided the use of a vaccine or immunogenic composition comprising a *Streptococcus pneumoniae* antigen, and an adjuvant composition comprising an oil-in-water emulsion wherein said oil-in-water emulsion comprises 0.5 - 10 mg metabolisable oil, 0.5 – 11 mg tocol and 0.4 – 4 mg emulsifying agent in the manufacture of an immunogenic composition for the treatment, alleviation or prevention of pneumococcal infection or disease.

In a further aspect, there is provided a method or use as hereinabove defined, for protection against infection or disease caused by a pathogen which is a variant of the pathogen from which the antigen in the immunogenic composition is derived. In another embodiment, there is provided a method or use as hereinabove defined for protection against infections or disease caused by a pathogen which comprises an antigen which is a variant of that antigen in the immunogenic composition.

BRIEF DESCRIPTION OF FIGURES

Figure 1: Clinical trial: geometric mean titers (GMTs) for anti-HA antibody at different timepoints (ATP cohort for immunogenicity).

Figure 2: Clinical trial: seroprotection rate (SPR) for HI antibody titer with 95% confidence interval at day 0 and day 21 (ATP cohort for immunogenicity).

Figure 3: Clinical trial: seroconversion rate (SCR) for HI antibody titer with 95% confidence interval at day 21 (ATP cohort for immunogenicity).

Figure 4: Clinical trial: seroconversion factor (SCF) for HI antibody titer with 95% confidence interval at day 21 (ATP cohort for immunogenicity).

Figure 5: Mice study: Haemagglutinin Inhibition test (GMT +/- IC95) in BALB/c mice primed with heterosubtypic strains (dose range AS03). Figure 5A: Anti-A/New Caledonia/20/99 HI titers; Figure 5B: Anti-A/Panama/2007/99 HI titers. Figure 5C: Anti-B/Shandong/7/97 HI titers.

Figure 6: Mice study: Haemagglutinin Inhibition test (GMT +/- IC95) in C57Bl/6 mice primed with heterosubtypic strains (dose range AS03).

Figure 7: Mice study: Cellular immune response (CD4+ T cell) in PBMC from C57Bl/6 mice primed with heterosubtypic strains (dose range AS03).

Figure 8: Mice study: Cellular immune response (CD4+ T cell) in PBMC from C57Bl/6 mice primed with heterosubtypic strains and immunized with low dose antigen (0.5 µg) adjuvanted with dose range AS03.

Figure 9: Mice study: H5N1-specific serum Ig ELISA titers (A and B) and anti-H5N1 IgG1 (C and D) and IgG2b (E and F) isotypic responses on day 14 post-immunization (GMT +/- IC95) for two different antigen dose: 1.5 µg (A, C and E) or 0.38 µg (B, D and F)

Figure 10: Mice study: Hemagglutination inhibition test (GMT +/- IC95) on day 21 post-immunization (GMT +/- IC95) for two different antigen dose: 1.5 µg (A) or 0.38 µg (B).

Figure 11: Mice study: Cellular immune response (CD4+ T cell) in naïve C57Bl/6 mice immunized with different dose of H5N1 vaccine (1.5 or 0.38 µg) adjuvanted with dose range AS03: (A) 1.5 µg HA Ag (antigen) or (B) 0.38 µg HA Ag (antigen).

Figure 12: Pigs study. Haemagglutinin Inhibition test (GMT +/- IC95) in pigs primed with homologous strains (dose range AS03).

Figure 13: Mice study. Humoral immune response measured by ELISA in C57bl mice immunised with 3µg of each of dPly, PhtD and Protein D in AS03 adjuvant containing 250, 125 or 62.5µg of SB62.

Figure 14: Mice study. Humoral immune response measured by ELISA in C57bl mice immunised with 6, 3 or 1.5µg of dPly, PhtD and Protein D in AS03 adjuvant containing 250, 125 or 62.5µg of SB62.

DETAILED DESCRIPTION OF THE INVENTION

The terms “comprising”, “comprise” and “comprises” herein are intended by the inventors to be optionally substitutable with the terms “consisting of”, “consist of” and “consists of”, respectively, in every instance.

Embodiments herein relating to “vaccine compositions” of the invention are also applicable to embodiments relating to “immunogenic compositions” of the invention, and vice versa.

In one embodiment of the invention there is provided a vaccine or immunogenic composition comprising an antigen or antigen composition and an adjuvant composition consisting of an oil in water emulsion, wherein said oil in water emulsion comprises 0.5 - 10 mg metabolisable oil, 0.5 – 11 mg tocol and 0.4 – 4 mg emulsifying agent, per human dose.

In a further embodiment of the invention there is provided a vaccine or immunogenic composition comprising an antigen or antigen composition and an adjuvant composition comprising an oil in water emulsion, wherein the oil in water emulsion comprises 0.5 - 10 mg metabolisable oil, (such as squalene), 0.5 – 11 mg tocol (such as alpha-tocopherol and 0.4 – 4 mg emulsifying agent (such as polyoxyethylene sorbitan monooleate), per human dose.

Oil in water emulsion component

The adjuvant composition of the invention comprises an oil-in-water emulsion adjuvant, preferably said emulsion comprises a metabolisable oil in an amount of 0.5-10mg, a tocol in an amount of 0.5 – 11 mg and an emulsifying agent in an amount of 0.4 – 4 mg and having oil droplets of which at least 70% by intensity have diameters of less than 1 µm.

In order for any oil in water composition to be suitable for human administration, the oil phase of the emulsion system has to comprise a metabolisable oil. The meaning of the term metabolisable oil is well known in the art. Metabolisable can be defined as ‘being capable of being transformed by metabolism’ (Dorland’s Illustrated Medical Dictionary, W.B. Sanders Company, 25th edition (1974)). The oil may be any vegetable oil, fish oil, animal oil or synthetic oil, which is not toxic to the recipient and is capable of being transformed by metabolism. Nuts, seeds, and grains are common sources of vegetable oils. Synthetic oils are also part of this invention and can include commercially available oils such as NEOBEE® and others. A particularly suitable metabolisable oil is squalene.

Squalene (2,6,10,15,19,23-Hexamethyl-2,6,10,14,18,22-tetracosahexaene) is an unsaturated oil which is found in large quantities in shark-liver oil, and in lower quantities in olive oil, wheat germ oil, rice bran oil, and yeast, and is a particularly preferred oil for use in this invention. Squalene is a metabolisable oil by virtue of the fact that it is an intermediate in the biosynthesis of cholesterol (Merck index, 10th Edition, entry no.8619).

Suitably the metabolisable oil is present in the adjuvant composition in an amount of 0.5-10 mg, preferably 1-10, 2-10, 3-9, 4-8, 5-7, or 5-6 mg (e.g. 2-3, 5-6, or 9-10mg), specifically 5.35 mg or 2.14 mg. In a further embodiment of the invention, the metabolisable oil is present in the vaccine (or immunogenic) composition in an amount of 0.5-10 mg, preferably 1-10, 2-10, 3-9, 4-8, 5-7, or 5-6 mg (e.g. 2-3, 5-6, or 9-10mg), specifically 5.35 mg or 2.14 mg.

The amount of metabolisable oil in vaccine or immunogenic composition may be expressed as a percentage of the total composition. Suitably the metabolisable oil is present in the vaccine composition in an amount of 0.5% to 2%, preferably 0.25 – 2, or 0.25-1.75, or 0.5-1.65, or 0.6-1.5, or 0.8 -1.4 or 1-1.25 % (v/v) oil of the total composition volume.

In another specific embodiment, the metabolisable oil is present in a final amount of about 1.25% of the total volume of the vaccine (or immunogenic) composition. In another specific embodiment, the metabolisable oil is present in a final amount of 0.25% (v/v) of the total composition volume.

By way of clarification, concentrations given in v/v can be converted into concentration in w/v by applying the following conversion factor: a 5% (v/v) squalene concentration is equivalent to a 4.28% (w/v) squalene concentration.

The oil in water emulsion comprises a tocol. Tocols are well known in the art and are described in EP0382271. Suitably the tocol is alpha-tocopherol or a derivative thereof such as alpha-tocopherol succinate (also known as vitamin E succinate). Said tocol is suitably present in the adjuvant composition in an amount of 0.5-11 mg, preferably 1-11, 2-10, 3-9, 4-8, 5-7, 5-6 (e.g. 10-11, 5-6, 2.5-3.5 or 1-3 mg). In a specific embodiment the tocol is present in an amount of 5.94 mg or 2.38 mg. In a further embodiment, said tocol is suitably present in the vaccine (or immunogenic) composition in an amount of 0.5-11

mg, preferably 1-11, 2-10, 3-9, 4-8, 5-7, 5-6 (e.g. 10-11, 5-6, 2.5-3.5 or 1-3 mg). In a specific embodiment the tocol is present in an amount of 5.94 mg or 2.38 mg.

The amount of tocol may be expressed as a percentage of the total vaccine or immunogenic composition volume. Suitably tocol is present in the vaccine composition in an amount 0.25% to 2% (v/v) of the total volume of the immunogenic composition, preferably at 0.25-2 comprises 0.25 – 2, or 0.25-1.75, or 0.5-1.65, or 0.6-1.5, or 0.8 -1.4 or 1-1.25 % (v/v) tocol of the total volume.

Preferably tocol is present in an amount of between 0.2% and 2% (v/v) of the total volume of the vaccine (or immunogenic) composition, more preferably at an amount of 1.25% (v/v) in a 0.5 ml dose volume.

In a specific embodiment, the tocol is present in a final amount of about 1.25% of the total volume of the vaccine or immunogenic composition. In another specific embodiment, the tocol is present in a final amount of 0.25% (v/v) of the total volume or 1.25% (v/v) in 0.5 ml dose volume or 0.9% (v/v), in 0.7 ml dose volume, or 0.5% (v/v) in 0.5ml dose or 0.35-0.37 %, preferably 0.36% in 0.7ml vaccine or immunogenic dose.

By way of clarification, concentrations given in v/v can be converted into concentration in w/v by applying the following conversion factor: a 5% (v/v) alpha-tocopherol concentration is equivalent to a 4.8% (w/v) alpha-tocopherol concentration.

The oil in water emulsion further comprises an emulsifying agent. The emulsifying agent may suitably be polyoxyethylene sorbitan monooleate. In a particular embodiment the emulsifying agent may be selected from the group comprising: Polysorbate® 80 or Tween® 80.

Said emulsifying agent is suitably present in the adjuvant composition in an amount of 0.1-5, 0.2-5, 0.3-4, 0.4-3 or 2-3 mg (e.g. 0.4-1.2, 2-3 or 4-5 mg) emulsifying agent. In a specific embodiment the emulsifying agent is present in an amount of 0.97 mg or 2.425 mg.

Further, said emulsifying agent is suitably present in the vaccine or immunogenic composition in an amount of 0.1-5, 0.2-5, 0.3-4, 0.4-3 or 2-3 mg (e.g. 0.4-1.2, 2-3 or 4-5

mg) emulsifying agent. In a specific embodiment the emulsifying agent is present in an amount of 0.97 mg or 2.425 mg.

The amount of emulsifying agent may be expressed as a percentage of the total vaccine or immunogenic composition volume. Suitably the emulsifying agent is present in the vaccine (or immunogenic) composition in an amount 0.125-0.8% (v/v) of the total volume of the composition, preferably at 0.08-.05, or 0.1-0.7, or 0.2-0.6, or 0.25-0.55, or 0.3-0.52 or 0.4-0.5% (v/v) of the total volume. In a specific embodiment the emulsifying agent is present in an amount of 1%, 0.5% or 0.2% (v/v) of the total vaccine or immunogenic composition volume.

By way of clarification, concentrations given in v/v can be converted into concentration in w/v by applying the following conversion factor: a 1.8% (v/v) polysorbate 80 concentration is equivalent to a 1.91% (w/v) polysorbate 80 concentration.

In a specific embodiment, a 0.5 ml vaccine or immunogenic dose volume contains 0.45% (v/v) Tween 80, and a 0.7 ml dose volume contains 0.315% (v/v) Tween 80. In another specific embodiment a 0.5 ml dose contains 0.18% (v/v) emulsifying agent and a 0.7 ml vaccine or immunogenic composition dose contains 0.126% (v/v) emulsifying agent.

By the term “human dose” is meant a dose which is in a volume suitable for human use. Generally this is between 0.25 and 1.5 ml. In one embodiment, a human dose is 0.5 ml. In a further embodiment, a human dose is higher than 0.5 ml, for example 0.6, 0.7, 0.8, 0.9 or 1 ml. In a further embodiment, a human dose is between 1 ml and 1.5 ml. In another embodiment, in particular when the immunogenic composition is for the paediatric population, a human dose may be less than 0.5 ml such as between 0.25 and 0.5 ml. The invention is characterised in that each or all of the individual components of the adjuvant within the immunogenic composition is/are at a lower level than previously thought useful and is/are typically as recited above. Particularly suitable compositions comprise the following adjuvant components in the following amounts are in a final volume of human dose of 0.5 ml:

Table 1

	Adjuvant A	Adjuvant B	Adjuvant E	Adjuvant F	Adjuvant C	Adjuvant G	Adjuvant D
o/w emulsion	125 µl	100 µl	83.33 µl	62.5 µl	50 µl	31.25 µl	25 µl

Components:							
Tocopherol	5.94 mg	4.28 mg	3.57 mg	2.68 mg	2.38 mg	1.34 mg	1.19 mg
Squalene	5.35 mg	4.75 mg	3.96 mg	2.97 mg	2.14 mg	1.49 mg	1.07 mg
Polysorbate 80	2.43 mg	1.94 mg	1.62 mg	1.21 mg	0.97 mg	0.61 mg	0.48 mg

The invention further provides an adjuvant composition comprising the individual components as defined herein above and in the amount defined above, for example but not exclusively as illustrated in Table 1. Typically such an adjuvant composition will be in a human dose suitable volume. Where the adjuvant is in a liquid form to be combined with a liquid form of an antigenic composition, the adjuvant composition will be in a human dose suitable volume which is a fraction of the intended final volume of the human dose, such as for example approximately half of the intended final volume of the human dose, for example a 350 µl volume for an intended human dose of 0.7ml, or a 250 µl volume for an intended human dose of 0.5 ml. The adjuvant composition is diluted when combined with the antigen composition to provide the final human dose of vaccine. The final volume of such dose will of course vary dependent on the initial volume of the adjuvant composition and the volume of antigen composition added to the adjuvant composition. In an alternative embodiment, liquid adjuvant is used to reconstitute a lyophilised antigen composition. In this embodiment, the human dose suitable volume of the adjuvant composition is approximately equal to the final volume of the human dose. The liquid adjuvant composition is added to the vial containing the lyophilised antigen composition. The final human dose can vary between 0.5 and 1.5 ml.

The method of producing oil-in-water emulsions is well known to the person skilled in the art. Commonly, the method comprises mixing the tocol-containing oil phase with a surfactant such as a PBS/TWEEN80™ solution, followed by homogenisation using a homogenizer, it would be clear to a man skilled in the art that a method comprising passing the mixture twice through a syringe needle would be suitable for homogenising small volumes of liquid. Equally, the emulsification process in microfluidiser (M110S Microfluidics machine, maximum of 50 passes, for a period of 2 minutes at maximum pressure input of 6 bar (output pressure of about 850 bar)) could be adapted by the man skilled in the art to produce smaller or larger volumes of emulsion. The adaptation could be achieved by routine experimentation comprising the measurement of the resultant emulsion until a preparation was achieved with oil droplets of the required diameter.

In an oil in water emulsion, the oil and emulsifier should be in an aqueous carrier. The aqueous carrier may be, for example, phosphate buffered saline.

Preferably the oil-in-water emulsion systems of the present invention have a small oil droplet size in the sub-micron range. Suitably the droplet sizes will be in the range 120 to 750 nm, more preferably sizes from 120 to 600 nm in diameter. Most preferably the oil-in water emulsion contains oil droplets of which at least 70% by intensity are less than 500 nm in diameter, more preferably at least 80% by intensity are less than 300 nm in diameter, more preferably at least 90% by intensity are in the range of 120 to 200 nm in diameter.

The oil droplet size, i.e. diameter, according to the present invention is given by intensity. There are several ways of determining the diameter of the oil droplet size by intensity. Intensity is measured by use of a sizing instrument, suitably by dynamic light scattering such as the Malvern Zetasizer 4000 or preferably the Malvern Zetasizer 3000HS. A detailed procedure is given in Example II.2. A first possibility is to determine the z average diameter ZAD by dynamic light scattering (PCS-Photon correlation spectroscopy); this method additionally give the polydispersity index (PDI), and both the ZAD and PDI are calculated with the cumulants algorithm. These values do not require the knowledge of the particle refractive index. A second mean is to calculate the diameter of the oil droplet by determining the whole particle size distribution by another algorithm, either the Contin, or NNLS, or the automatic "Malvern" one (the default algorithm provided for by the sizing instrument). Most of the time, as the particle refractive index of a complex composition is unknown, only the intensity distribution is taken into consideration, and if necessary the intensity mean originating from this distribution.

Optional Immunostimulants

In a further embodiment of the invention there is provided a vaccine or immunogenic composition comprising an antigen or antigen composition and an adjuvant composition comprising an oil in water emulsion and optionally one or more further immunostimulants, wherein said oil in water emulsion comprises 0.5 - 10 mg metabolisable oil, 0.5 – 11 mg tocol and 0.4 – 4 mg emulsifying agent.

In one embodiment the adjuvant composition comprises an oil and water emulsion as described herein. In a further embodiment the adjuvant composition may further comprise

one or more additional adjuvants or immunostimulants. In a further embodiment the adjuvant composition optionally comprises one or more additional adjuvants or immunostimulants other than QS21 and/or MPL.

The optional additional adjuvant is selected from the group: a saponin, lipid A or a derivative thereof, an immunostimulatory oligonucleotide, an alkyl glucosaminide phosphate, a metal salt, a toll-like receptor agonist or combinations thereof. It is preferred that the adjuvant is a Toll like receptor agonist in particular an agonist of a Toll like receptor 2, 3, 4, 7, 8 or 9, or a saponin. It is further preferred that the adjuvant system comprises two or more adjuvants from the above list. Combinations preferably contain a saponin (in particular QS21) adjuvant and/or a Toll like receptor 4 agonist such as 3D-MPL or a Toll like recpetor 9 agonist such as a CpG containing immunostimulatory oligonucleotide. Other preferred combinations comprise a saponin (in particular QS21) and a Toll like receptor 4 agonist such as a saponin (in particular QS21) and a Toll like receptor 4 ligand such as 3D-MPL or an alkyl glucosaminide phosphate.

In an embodiment the additional adjuvant is a Toll like receptor (TLR) 4 ligand, preferably an agonist such as a lipid A derivative particularly monophosphoryl lipid A or more particularly 3 Deacylated monophoshoryl lipid A (3 D – MPL).

3D–MPL is available under the trademark MPL® by GlaxoSmithKline Biologicals North America and primarily promotes CD4+ T cell responses with an IFN-g (Th1) phenotype. It can be produced according to the methods disclosed in GB 2 220 211 A. Chemically it is a mixture of 3-deacylated monophosphoryl lipid A with 3, 4, 5 or 6 acylated chains. Preferably in the compositions of the present invention small particle 3 D- MPL is used. Small particle 3 D -MPL has a particle size such that it may be sterile-filtered through a 0.22μm filter. Such preparations are described in International Patent Application No. WO 94/21292. Synthetic derivatives of lipid A are known and thought to be TLR 4 agonists including, but not limited to:

OM174 (2-deoxy-6-o-[2-deoxy-2-[(R)-3-dodecanoyloxytetra-decanoylamino]-4-o-phosphono-β-D-glucopyranosyl]-2-[(R)-3-hydroxytetradecanoylamino]-α-D-glucopyranosyldihydrogenphosphate), (WO 95/14026)

OM 294 DP (3S, 9 R) –3--[(R)-dodecanoyloxytetradecanoylamino]-4-oxo-5-aza-9(R)-[(R)-3-hydroxytetradecanoylamino]decan-1,10-diol,1,10-bis(dihydrogenophosphate) (WO99 /64301 and WO 00/0462)

OM 197 MP-Ac DP (3S-, 9R) -3-[(R) -dodecanoyloxytetradecanoylamino]-4-oxo-5-aza-9-[(R)-3-hydroxytetradecanoylamino]decan-1,10-diol,1 -dihydrogenophosphate 10-(6-aminohexanoate) (WO 01/46127)

Other TLR4 ligands which may be used are alkyl Glucosaminide phosphates (AGPs) such as those disclosed in WO9850399 or US6303347 (processes for preparation of AGPs are also disclosed), or pharmaceutically acceptable salts of AGPs as disclosed in US6764840. Some AGPs are TLR4 agonists, and some are TLR4 antagonists. Both are thought to be useful as adjuvants.

Other suitable TLR-4 ligands, capable of causing a signalling response through TLR-4 (Sabroe et al, JI 2003 p1630-5) are, for example, lipopolysaccharide from gram-negative bacteria and its derivatives, or fragments thereof, in particular a non-toxic derivative of LPS (such as 3D-MPL). Other suitable TLR agonist are: heat shock protein (HSP) 10, 60, 65, 70, 75 or 90; surfactant Protein A, hyaluronan oligosaccharides, heparan sulphate fragments, fibronectin fragments, fibrinogen peptides and b-defensin-2, muramyl dipeptide (MDP) or F protein of respiratory syncitial virus. In one embodiment the TLR agonist is HSP 60, 70 or 90.

Toll-like receptors (TLRs) are type I transmembrane receptors, evolutionarily conserved between insects and humans. Ten TLRs have so far been established (TLRs 1-10) (Sabroe et al, JI 2003 p1630-5). Members of the TLR family have similar extracellular and intracellular domains; their extracellular domains have been shown to have leucine – rich repeating sequences, and their intracellular domains are similar to the intracellular region of the interleukin – 1 receptor (IL-1R). TLR cells are expressed differentially among immune cells and other cells (including vascular epithelial cells, adipocytes, cardiac myocytes and intestinal epithelial cells). The intracellular domain of the TLRs can interact with the adaptor protein Myd88, which also posses the IL-1R domain in its cytoplasmic region, leading to NF-KB activation of cytokines; this Myd88 pathway is one way by which cytokine release is effected by TLR activation. The main expression of TLRs is in cell types such as antigen presenting cells (eg dendritic cells, macrophages etc).

Activation of dendritic cells by stimulation through the TLRs leads to maturation of dendritic cells, and production of inflammatory cytokines such as IL-12. Research carried out so far has found that TLRs recognise different types of agonists, although some agonists are common to several TLRs. TLR agonists are predominantly derived from bacteria or viruses, and include molecules such as flagellin or bacterial lipopolysaccharide (LPS).

By "TLR agonist" it is meant a component which is capable of causing a signalling response through a TLR signalling pathway, either as a direct ligand or indirectly through generation of endogenous or exogenous ligand (Sabroe et al, JI 2003 p1630-5).

In another embodiment, other natural or synthetic agonists of TLR molecules are used as optional additional immunostimulants. These could include, but are not limited to agonists for TLR2, TLR3, TLR7, TLR8 and TLR9.

In one embodiment of the present invention, a TLR agonist is used that is capable of causing a signalling response through TLR-1 (Sabroe et al, JI 2003 p1630-5). Suitably, the TLR agonist capable of causing a signalling response through TLR-1 is selected from: Tri-acylated lipopeptides (LPs); phenol-soluble modulin; Mycobacterium tuberculosis LP; S-(2,3-bis(palmitoyloxy)-(2-RS)-propyl)-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys(4)-OH, trihydrochloride (Pam_3Cys) LP which mimics the acetylated amino terminus of a bacterial lipoprotein and OspA LP from Borrelia burgdorfei.

In an alternative embodiment, a TLR agonist is used that is capable of causing a signalling response through TLR-2 (Sabroe et al, JI 2003 p1630-5). Suitably, the TLR agonist capable of causing a signalling response through TLR-2 is one or more of a lipoprotein, a peptidoglycan, a bacterial lipopeptide from M tuberculosis, B burgdorferi. T pallidum; peptidoglycans from species including Staphylococcus aureus; lipoteichoic acids, mannuronic acids, Neisseria porins, bacterial fimbriae, Yersina virulence factors, CMV virions, measles haemagglutinin, and zymosan from yeast.

In an alternative embodiment, a TLR agonist is used that is capable of causing a signalling response through TLR-3 (Sabroe et al, JI 2003 p1630-5). Suitably, the TLR agonist capable of causing a signalling response through TLR-3 is double stranded RNA

(dsRNA), or polyinosinic-polycytidylic acid (Poly IC), a molecular nucleic acid pattern associated with viral infection.

In an alternative embodiment, a TLR agonist is used that is capable of causing a signalling response through TLR-5 (Sabroe et al, JI 2003 p1630-5). Suitably, the TLR agonist capable of causing a signalling response through TLR-5 is bacterial flagellin.

In an alternative embodiment, a TLR agonist is used that is capable of causing a signalling response through TLR-6 (Sabroe et al, JI 2003 p1630-5). Suitably, the TLR agonist capable of causing a signalling response through TLR-6 is mycobacterial lipoprotein, di-acylated LP, and phenol-soluble modulin. Further TLR6 agonists are described in WO2003043572.

In an alternative embodiment, a TLR agonist is used that is capable of causing a signalling response through TLR-7 (Sabroe et al, JI 2003 p1630-5). Suitably, the TLR agonist capable of causing a signalling response through TLR-7 is a single stranded RNA (ssRNA), loxoribine, a guanosine analogue at positions N7 and C8, or an imidazoquinoline compound, or derivative thereof. In one embodiment, the TLR agonist is imiquimod. Further TLR7 agonists are described in WO02085905.

In an alternative embodiment, a TLR agonist is used that is capable of causing a signalling response through TLR-8 (Sabroe et al, JI 2003 p1630-5). Suitably, the TLR agonist capable of causing a signalling response through TLR-8 is a single stranded RNA (ssRNA), an imidazoquinoline molecule with anti-viral activity, for example resiquimod (R848); resiquimod is also capable of recognition by TLR-7. Other TLR-8 agonists which may be used include those described in WO2004071459.

Immunostimulatory oligonucleotides or any other Toll-like receptor (TLR) 9 agonist may also be used. The preferred oligonucleotides for use in adjuvants or vaccines or immunogenic compositions of the present invention are CpG containing oligonucleotides, preferably containing two or more dinucleotide CpG motifs separated by at least three, more preferably at least six or more nucleotides. A CpG motif is a Cytosine nucleotide followed by a Guanine nucleotide. The CpG oligonucleotides of the present invention are typically deoxynucleotides. In a preferred embodiment the internucleotide in the oligonucleotide is phosphorodithioate, or more preferably a phosphorothioate bond, although phosphodiester and other internucleotide bonds are within the scope of the

invention. Also included within the scope of the invention are oligonucleotides with mixed internucleotide linkages. Methods for producing phosphorothioate oligonucleotides or phosphorodithioate are described in US5,666,153, US5,278,302 and WO95/26204.

Examples of preferred oligonucleotides have the following sequences. The sequences preferably contain phosphorothioate modified internucleotide linkages.

OLIGO 1(SEQ ID NO:1): TCC ATG ACG TTC CTG ACG TT (CpG 1826)
OLIGO 2 (SEQ ID NO:2): TCT CCC AGC GTG CGC CAT (CpG 1758)
OLIGO 3(SEQ ID NO:3): ACC GAT GAC GTC GCC GGT GAC GGC ACC ACG
OLIGO 4 (SEQ ID NO:4): TCG TCG TTT TGT CGT TTT GTC GTT (CpG 2006)
OLIGO 5 (SEQ ID NO:5): TCC ATG ACG TTC CTG ATG CT (CpG 1668)
OLIGO 6 (SEQ ID NO:6): TCG ACG TTT TCG GCG CGC GCC G (CpG 5456)

Alternative CpG oligonucleotides may comprise the preferred sequences above in that they have inconsequential deletions or additions thereto. The CpG oligonucleotides utilised in the present invention may be synthesized by any method known in the art (for example see EP 468520). Conveniently, such oligonucleotides may be synthesized utilising an automated synthesizer.

Accordingly, in another embodiment, the adjuvant composition further comprises an additional immunostimulant which is selected from the group consisting of: a TLR-1 agonist, a TLR-2 agonist, TLR-3 agonist, a TLR-4 agonist, TLR-5 agonist, a TLR-6 agonist, TLR-7 agonist, a TLR-8 agonist, TLR-9 agonist, or a combination thereof.

Another preferred immunostimulants for use in the present invention is Quil A and its derivatives. Quil A is a saponin preparation isolated from the South American tree *Quilaja Saponaria Molina* and was first described as having adjuvant activity by Dalsgaard *et al*. in 1974 ("Saponin adjuvants", Archiv. für die gesamte Virusforschung, Vol. 44, Springer Verlag, Berlin, p243-254). Purified fragments of Quil A have been isolated by HPLC which retain adjuvant activity without the toxicity associated with Quil A (EP 0 362 278), for example QS7 and QS21 (also known as QA7 and QA21). QS-21 is a natural saponin derived from the bark of *Quillaja saponaria* Molina which induces CD8+ cytotoxic T cells (CTLs), Th1 cells and a predominant IgG2a antibody response and is a preferred saponin in the context of the present invention.

Particular formulations of QS21 have been described which are particularly preferred, these formulations further comprise a sterol (WO96/33739). Where squalene and a saponin (optionally QS21) are included, it is of benefit to also include a sterol (optionally cholesterol) to the formulation as this allows a reduction in the total level of oil in the emulsion. This leads to a reduced cost of manufacture, improvement of the overall comfort of the vaccination, and also qualitative and quantitative improvements of the resultant immune responses, such as improved IFN-γ production. Accordingly, the adjuvant system of the present invention typically comprises a ratio of metabolisable oil:saponin (w/w) in the range of 200:1 to 300:1, also the present invention can be used in a "low oil" form the optional range of which is 1:1 to 200:1, optionally 20:1 to 100:1, or substantially 48:1, this vaccine retains the beneficial adjuvant properties of all of the components, with a much reduced reactogenicity profile. Accordingly, some embodiments have a ratio of squalene:QS21 (w/w) in the range of 1:1 to 250:1, or 20:1 to 200:1, or 20:1 to 100:1, or substantially 48:1. Optionally a sterol (e.g. cholesterol) is also included present at a ratio of saponin:sterol as described herein.

Antigens and antigen composition

The vaccine or immunogenic formulations contain a *Streptococcus pneumoniae* antigen capable of eliciting an immune response against a human or animal pathogen. Where the pneumococcal antigen is a protein, the protein is optionally conjugation for example to a saccharide. Optionally, the protein is unconjugated or present in the immunogenic composition as a free protein. An unconjugated protein is not covalently bound to a saccharide as a carrier protein.

In an embodiment of the invention, the immunogenic compositon of the invention comprises a pneumococcal protein which is optionally a member of the polyhistidine triad family (Pht) proteins, fragments or fusion proteins or immunologically functional equivalent thereof. The PhtA, PhtB, PhtD or PhtE proteins may have an amino acid sequence sharing 80%, 85%, 90%, 95%, 98%, 99% or 100% identity with a sequence disclosed in WO 00/37105 or WO 00/39299 (e.g. with amino acid sequence 1-838 or 21-838 of SEQ ID NO: 4 of WO 00/37105 for PhtD). For example, fusion proteins are composed of full length or fragments of 2, 3 or 4 of PhtA, PhtB, PhtD, PhtE. Examples of fusion proteins are PhtA/B, PhtA/D, PhtA/E, PhtB/A, PhtB/D, PhtB/E. PhtD/A. PhtD/B, PhtD/E, PhtE/A,

PhtE/B and PhtE/D, wherein the proteins are linked with the first mentioned at the N-terminus (see for example WO01/98334).

Where fragments of Pht proteins are used (separately or as part of a fusion protein), each fragment optionally contains one or more histidine triad motif(s) and/or coiled coil regions of such polypeptides. A histidine triad motif is the portion of polypeptide that has the sequence HxxHxH where H is histidine and x is an amino acid other than histidine. A coiled coil region is a region predicted by "Coils" algorithm Lupus, A et al (1991) Science 252; 1162-1164. In an embodiment the or each fragment includes one or more histidine triad motif as well as at least one coiled coil region. In an embodiment, the or each fragment contains exactly or at least 2, 3, 4 or 5 histidine triad motifs (optionally, with native Pht sequence between the 2 or more triads, or intra-triad sequence that is more than 50, 60, 70, 80, 90 or 100 % identical to a native pneumococcal intra-triad Pht sequence – e.g. the intra-triad sequence shown in SEQ ID NO: 4 of WO 00/37105 for PhtD). In an embodiment, the or each fragment contains exactly or at least 2, 3 or 4 coiled coil regions. In an embodiment a Pht protein disclosed herein includes the full length protein with the signal sequence attached, the mature full length protein with the signal peptide (for example 20 amino acids at N-terminus) removed, naturally occurring variants of Pht protein and immunogenic fragments of Pht protein (e.g. fragments as described above or polypeptides comprising at least 15 or 20 contiguous amino acids from an amino acid sequence in WO00/37105 or WO00/39299 wherein said polypeptide is capable of eliciting an immune response specific for said amino acid sequence in WO00/37105 or WO00/39299).

In particular, the term "PhtD" as used herein includes the full length protein with the signal sequence attached, the mature full length protein with the signal peptide (for example 20 amino acids at N-terminus) removed, naturally occurring variants of PhtD and immunogenic fragments of PhtD (e.g. fragments as described above or polypeptides comprising at least 15 or 20 contiguous amino acids from a PhtD amino acid sequence in WO00/37105 or WO00/39299 wherein said polypeptide is capable of eliciting an immune response specific for said PhtD amino acid sequence in WO00/37105 or WO00/39299 (e.g. SEQ ID NO: 4 of WO 00/37105 for PhtD).

In one embodiment, the immunogenic composition of the invention comprises at least 1 protein selected from the group consisting of the Poly Histidine Triad family (PhtX), Choline Binding Protein family (CbpX), CbpX truncates, LytX family, LytX truncates, CbpX

truncate-LytX truncate chimeric proteins (or fusions), pneumolysin (Ply), PspA, PsaA, Sp128, Sp101, Sp130, Sp125 and Sp133. In a further embodiment, the immunogenic composition comprises 2 or more proteins selected from the group consisting of the Poly Histidine Triad family (PhtX), Choline Binding Protein family (CbpX), CbpX truncates, LytX family, LytX truncates, CbpX truncate-LytX truncate chimeric proteins (or fusions), pneumolysin (Ply), PspA, PsaA, and Sp128. In one more embodiment, the immunogenic composition comprises 2 or more proteins selected from the group consisting of the Poly Histidine Triad family (PhtX), Choline Binding Protein family (CbpX), CbpX truncates, LytX family, LytX truncates, CbpX truncate-LytX truncate chimeric proteins (or fusions), pneumolysin (Ply), and Sp128.

The Pht (Poly Histidine Triad) family comprises proteins PhtA, PhtB, PhtD, and PhtE. The family is characterized by a lipidation sequence, two domains separated by a proline-rich region and several histidine triads, possibly involved in metal or nucleoside binding or enzymatic activity, (3-5) coiled-coil regions, a conserved N-terminus and a heterogeneous C terminus. It is present in all strains of pneumococci tested. Homologous proteins have also been found in other Streptococci and Neisseria. In one embodiment of the invention, the Pht protein of the invention is PhtD. It is understood, however, that the terms Pht A, B, D, and E refer to proteins having sequences disclosed in the citations below as well as naturally-occurring (and man-made) variants thereof that have a sequence homology that is at least 90% identical to the referenced proteins. Optionally it is at least 95% identical or at least 97% identical.

With regards to the PhtX proteins, PhtA is disclosed in WO 98/18930, and is also referred to Sp36. As noted above, it is a protein from the polyhistidine triad family and has the type II signal motif of LXXC. PhtD is disclosed in WO 00/37105, and is also referred to Sp036D. As noted above, it also is a protein from the polyhistidine triad family and has the type II LXXC signal motif. PhtB is disclosed in WO 00/37105, and is also referred to Sp036B. Another member of the PhtB family is the C3-Degrading Polypeptide, as disclosed in WO 00/17370. This protein also is from the polyhistidine triad family and has the type II LXXC signal motif. For example, an immunologically functional equivalent is the protein Sp42 disclosed in WO 98/18930. A PhtB truncate (approximately 79kD) is disclosed in WO99/15675 which is also considered a member of the PhtX family. PhtE is disclosed in WO00/30299 and is referred to as BVH-3. Where any Pht protein is referred to herein, it is meant that immunogenic fragments or fusions thereof of the Pht protein can be used. For example, a reference to PhtX includes immunogenic fragments or fusions

thereof from any Pht protein. A reference to PhtD or PhtB is also a reference to PhtDE or PhtBE fusions as found, for example, in WO0198334.

Pneumolysin is a multifunctional toxin with a distinct cytolytic (hemolytic) and complement activation activities (Rubins et al., Am . Respi. Cit Care Med, 153:1339-1346 (1996)). The toxin is not secreted by pneumococci, but it is released upon lysis of pneumococci under the influence of autolysin. Its effects include e.g., the stimulation of the production of inflammatory cytokines by human monocytes, the inhibition of the beating of cilia on human respiratory epithelial, and the decrease of bactericidal activity and migration of neutrophils. The most obvious effect of pneumolysin is in the lysis of red blood cells, which involves binding to cholesterol. Because it is a toxin, it needs to be detoxified (i.e., non-toxic to a human when provided at a dosage suitable for protection) before it can be administered *in vivo*. Expression and cloning of wild-type or native pneumolysin is known in the art. See, for example, Walker et al. (Infect Immun, 55:1184-1189 (1987)), Mitchell et al. (Biochim Biophys Acta, 1007:67-72 (1989) and Mitchell et al (NAR, 18:4010 (1990)). Detoxification of ply can be conducted by chemical means, e.g., subject to formalin or glutaraldehyde treatment or a combination of both (WO 04081515, PCT/EP2005/010258). Such methods are well known in the art for various toxins. Alternatively, ply can be genetically detoxified. Thus, the invention encompasses derivatives of pneumococcal proteins which may be, for example, mutated proteins. The term “mutated” is used herein to mean a molecule which has undergone deletion, addition or substitution of one or more amino acids using well known techniques for site directed mutagenesis or any other conventional method. For example, as described above, a mutant ply protein may be altered so that it is biologically inactive whilst still maintaining its immunogenic epitopes, see, for example, WO90/06951, Berry et al. (Infect Immun, 67:981-985 (1999)) and WO99/03884.

As used herein, it is understood that the term “Ply” refers to mutated or detoxified pneumolysin suitable for medical use (i.e., non toxic).

Concerning the Choline Binding Protein family (CbpX), members of that family were originally identified as pneumococcal proteins that could be purified by choline-affininty chromatography. All of the choline-binding proteins are non-covalently bound to phosphorylcholine moieties of cell wall teichoic acid and membrane-associated lipoteichoic acid. Structurally, they have several regions in common over the entire family, although the exact nature of the proteins (amino acid sequence, length, etc.) can vary. In

general, choline binding proteins comprise an N terminal region (N), conserved repeat regions (R1 and/or R2), a proline rich region (P) and a conserved choline binding region (C), made up of multiple repeats, that comprises approximately one half of the protein. As used in this application, the term "Choline Binding Protein family (CbpX)" is selected from the group consisting of Choline Binding Proteins as identified in WO97/41151, PbcA, SpsA, PspC, CbpA, CbpD, and CbpG. CbpA is disclosed in WO97/41151. CbpD and CbpG are disclosed in WO00/29434. PspC is disclosed in WO97/09994. PbcA is disclosed in WO98/21337.SpsA is a Choline binding protein disclosed in WO 98/39450. Optionally the Choline Binding Proteins are selected from the group consisting of CbpA, PbcA, SpsA and PspC.

An embodiment of the invention comprises CbpX truncates wherein "CbpX" is defined above and "truncates" refers to CbpX proteins lacking 50% or more of the Choline binding region (C). Optionally such proteins lack the entire choline binding region. Optionally, the such protein truncates lack (i) the choline binding region and (ii) a portion of the N-terminal half of the protein as well, yet retain at least one repeat region (R1 or R2). Optionally, the truncate has 2 repeat regions (R1 and R2). Examples of such embodiments are NR1xR2 and R1xR2 as illustrated in WO99/51266 or WO99/51188, however, other choline binding proteins lacking a similar choline binding region are also contemplated within the scope of this invention.

The LytX family is membrane associated proteins associated with cell lysis. The N-terminal domain comprises choline binding domain(s), however the LytX family does not have all the features found in the CbpA family noted above and thus for the present invention, the LytX family is considered distinct from the CbpX family. In contrast with the CbpX family, the C-terminal domain contains the catalytic domain of the LytX protein family. The family comprises LytA, B and C. With regards to the LytX family, LytA is disclosed in Ronda et al., Eur J Biochem, 164:621-624 (1987). LytB is disclosed in WO 98/18930, and is also referred to as Sp46. LytC is also disclosed in WO 98/18930, and is also referred to as Sp91. An embodiment of the invention comprises LytC.

Another embodiment comprises LytX truncates wherein "LytX" is defined above and "truncates" refers to LytX proteins lacking 50% or more of the Choline binding region. Optionally such proteins lack the entire choline binding region. Yet another embodiment of this invention comprises CbpX truncate-LytX truncate chimeric proteins (or fusions). Optionally this comprises NR1xR2 (or R1xR2) of CbpX and the C-terminal portion (Cterm,

i.e., lacking the choline binding domains) of LytX (e.g., LytCCterm or Sp91Cterm). Optionally CbpX is selected from the group consisting of CbpA, PbcA, SpsA and PspC. Optionally, it is CbpA. Optionally, LytX is LytC (also referred to as Sp91). Another embodiment of the present invention is a PspA or PsaA truncate lacking the choline binding domain (C) and expressed as a fusion protein with LytX. Optionally, LytX is LytC.

With regards to PsaA and PspA, both are know in the art. For example, PsaA and transmembrane deletion variants thereof have been described by Berry & Paton, Infect Immun 1996 Dec;64(12):5255-62. PspA and transmembrane deletion variants thereof have been disclosed in, for example, US 5804193, WO 92/14488, and WO 99/53940.

Sp128 and Sp130 are disclosed in WO00/76540. Sp125 is an example of a pneumococcal surface protein with the Cell Wall Anchored motif of LPXTG (where X is any amino acid). Any protein within this class of pneumococcal surface protein with this motif has been found to be useful within the context of this invention, and is therefore considered a further protein of the invention. Sp125 itself is disclosed in WO 98/18930, and is also known as ZmpB – a zinc metalloproteinase. Sp101 is disclosed in WO 98/06734 (where it has the reference # y85993). It is characterized by a Type I signal sequence. Sp133 is disclosed in WO 98/06734 (where it has the reference # y85992). It is also characterized by a Type I signal sequence.

The proteins of the invention may also be beneficially combined. By combined is meant that the immunogenic composition comprises all of the proteins from within the following combinations, either as carrier proteins or as free proteins or a mixture of the two. For example, in a combination of two proteins as set out hereinafter, both proteins may be used as carrier proteins, or both proteins may be present as free proteins, or both may be present as carrier and as free protein, or one may be present as a carrier protein and a free protein whilst the other is present only as a carrier protein or only as a free protein, or one may be present as a carrier protein and the other as a free protein. Where a combination of three proteins is given, similar possibilities exist. Combinations include, but are not limited to, PhtD + NR1xR2, PhtD + NR1xR2-Sp91Cterm chimeric or fusion proteins, PhtD + Ply, PhtD + Sp128, PhtD + PsaA, PhtD + PspA, PhtA + NR1xR2, PhtA + NR1xR2-Sp91Cterm chimeric or fusion proteins, PhtA + Ply, PhtA + Sp128, PhtA + PsaA, PhtA + PspA, NR1xR2 + LytC, NR1xR2 + PspA, NR1xR2 + PsaA, NR1xR2 + Sp128, R1xR2 + LytC, R1xR2 + PspA, R1xR2 + PsaA, R1xR2 + Sp128, R1xR2 + PhtD, R1xR2 +

PhtA. Optionally, NR1xR2 (or R1xR2) is from CbpA or PspC. Optionally it is from CbpA. Other combinations include 3 protein combinations such as PhtD + NR1xR2 + Ply, and PhtA + NR1xR2 + PhtD. In one embodiment, the vaccine composition comprises detoxified pneumolysin and PhtD or PhtDE as carrier proteins. In a further embodiment, the vaccine composition comprises detoxified pneumolysin and PhtD or PhtDE as free proteins.

An effective immune response against PhtD or fusion protein thereof is measured for example by a protection assay such as that described in example 15. An effective immune response provides at least 40%, 50%, 60%, 70%, 80% or 90% survival 7 days after challenge with a heterologous strain. Given that the challenge strain is heterologous, the protection afforded is due to the immune response against PhtD or fusion protein thereof.

Alternatively, an effective immune response against PhtD is measured by ELISA as described in example 14. An effective immune response gives an anti-PhtD IgG response of at least 250, 300, 350, 400, 500, 550 or 600 μg/ml GMC.

In an embodiment, the immunogenic composition of the invention comprises pneumolysin.

In an embodiment, the immunogenic composition of the invention comprises a pneumococcal protein disclosed in US6699703, for example, pneumococcal pprotein having the sequence or encoded by the sequence of SEQ ID NOs 1-5326 of US6699703.

In an embodiment of the invention, the immunogenic composition comprises a pneumococcal protein which is not disclosed in PCT/EP2007/060743.

The present invention further provides a vaccine containing the immunogenic compositions of the invention and a pharmaceutically acceptable excipient.

In an embodiment, the immunogenic composition of the invention comprises a pneumococcal protein selected from the group consisting of of PhtA, PhtD, PhtB, PhtE, LytB, LytC, LytA, Sp125, SP101, Sp128, Sp130, Sp133 or proteins disclosed in US6699703, including *S. pneumoniae* proteins represented or encoded by SEQ ID NOs: 5225, 5200, 3166, 4360, 5137, 4263, 3166, 5226, 3716, 4360, 5243, 3964, 5179, 3850, 4263, 5137, 5226, 5325, 3850, 5179 or 5325 of US6699703, or immunologically functional

equivalent thereof. Immunologically functional equivalents include sequences which share at least 80, 90, 95, 98 or 99% identity to the stated sequence. Immunologically functional equivalent also include fragments having at least 6, 10, 20, 30, 40 or 50 contiguous amino acids from the stated sequence. Immunologically functional equivalents are capable of eliciting an immune response which recognises the native antigen.

Vaccination

The vaccine preparations containing immunogenic compositions of the present invention may be used to protect or treat a mammal susceptible to infection, by means of administering said vaccine via systemic or mucosal route. These administrations may include injection *via* the intramuscular, intraperitoneal, intradermal or subcutaneous routes; or *via* mucosal administration to the oral/alimentary, respiratory, genitourinary tracts. Although the vaccine of the invention may be administered as a single dose, components thereof may also be co-administered together at the same time or at different times (for instance pneumococcal saccharide conjugates could be administered separately, at the same time or 1-2 weeks after the administration of the any bacterial protein component of the vaccine for optimal coordination of the immune responses with respect to each other). In addition, the vaccines of the invention may be administered IM for priming doses and IN for booster doses.

The content of protein antigens in the vaccine will typically be in the range 1-100μg, preferably 5-50μg, most typically in the range 5 - 25μg. Following an initial vaccination, subjects may receive one or several booster immunizations adequately spaced.

Vaccine preparation is generally described in Vaccine Design ("The subunit and adjuvant approach" (eds Powell M.F. & Newman M.J.) (1995) Plenum Press New York). Encapsulation within liposomes is described by Fullerton, US Patent 4,235,877.

The vaccines of the present invention may be stored in solution or lyophilized. Preferably the solution is lyophilized in the presence of a sugar such as sucrose or lactose. It is still further preferable that they are lyophilized and extemporaneously reconstituted prior to use.

In one aspect of the invention is provided a vaccine kit, comprising a vial containing an immunogenic composition of the invention, optionally in lyophilised form, and further

comprising a vial containing an adjuvant as described herein. It is envisioned that in this aspect of the invention, the adjuvant will be used to reconstitute the lyophilised immunogenic composition.

Although the vaccines of the present invention may be administered by any route, administration of the described vaccines into the skin (ID) forms one embodiment of the present invention. Human skin comprises an outer "horny" cuticle, called the stratum corneum, which overlays the epidermis. Underneath this epidermis is a layer called the dermis, which in turn overlays the subcutaneous tissue. Researchers have shown that injection of a vaccine into the skin, and in particular the dermis, stimulates an immune response, which may also be associated with a number of additional advantages. Intradermal vaccination with the vaccines described herein forms a preferred feature of the present invention.

The conventional technique of intradermal injection, the "mantoux procedure", comprises steps of cleaning the skin, and then stretching with one hand, and with the bevel of a narrow gauge needle (26-31 gauge) facing upwards the needle is inserted at an angle of between 10-15°. Once the bevel of the needle is inserted, the barrel of the needle is lowered and further advanced whilst providing a slight pressure to elevate it under the skin. The liquid is then injected very slowly thereby forming a bleb or bump on the skin surface, followed by slow withdrawal of the needle.

More recently, devices that are specifically designed to administer liquid agents into or across the skin have been described, for example the devices described in WO 99/34850 and EP 1092444, also the jet injection devices described for example in WO 01/13977; US 5,480,381, US 5,599,302, US 5,334,144, US 5,993,412, US 5,649,912, US 5,569,189, US 5,704,911, US 5,383,851, US 5,893,397, US 5,466,220, US 5,339,163, US 5,312,335, US 5,503,627, US 5,064,413, US 5,520, 639, US 4,596,556, US 4,790,824, US 4,941,880, US 4,940,460, WO 97/37705 and WO 97/13537. Alternative methods of intradermal administration of the vaccine preparations may include conventional syringes and needles, or devices designed for ballistic delivery of solid vaccines (WO 99/27961), or transdermal patches (WO 97/48440; WO 98/28037); or applied to the surface of the skin (transdermal or transcutaneous delivery WO 98/20734 ; WO 98/28037).

When the vaccines of the present invention are to be administered to the skin, or more specifically into the dermis, the vaccine is in a low liquid volume, particularly a volume of between about 0.05 ml and 0.2 ml.

The content of antigens in the skin or intradermal vaccines of the present invention may be similar to conventional doses as found in intramuscular vaccines (see above). However, it is a feature of skin or intradermal vaccines that the formulations may be "low dose". Accordingly the protein antigens in "low dose" vaccines are preferably present in as little as 0.1 to 10μg, preferably 0.1 to 5 μg per dose; and the saccharide (preferably conjugated) antigens may be present in the range of 0.01-1μg, and preferably between 0.01 to 0.5 μg of saccharide per dose.

As used herein, the term "intradermal delivery" means delivery of the vaccine to the region of the dermis in the skin. However, the vaccine will not necessarily be located exclusively in the dermis. The dermis is the layer in the skin located between about 1.0 and about 2.0 mm from the surface in human skin, but there is a certain amount of variation between individuals and in different parts of the body. In general, it can be expected to reach the dermis by going 1.5 mm below the surface of the skin. The dermis is located between the stratum corneum and the epidermis at the surface and the subcutaneous layer below. Depending on the mode of delivery, the vaccine may ultimately be located solely or primarily within the dermis, or it may ultimately be distributed within the epidermis and the dermis.

The amount of each antigen in each vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccinees. Such amount will vary depending upon which specific immunogen is employed and how it is presented.

In a further embodiment there is provided a method of treatment of an individual susceptible to or suffering from a disease by the administration of a composition as substantially described herein.

Also provided is a method to prevent an individual from contracting a disease selected from the group comprising infectious bacterial and viral diseases, parasitic diseases, particularly intracellular pathogenic disease, proliferative diseases such as prostate,

breast, colorectal, lung, pancreatic, renal, ovarian or melanoma cancers; non-cancer chronic disorders, allergy comprising the administration of a composition as substantially described herein to said individual.

In a further embodiment there is provided a vaccine composition for use in the prophylactic therapy or therapy of a condition or disease wherein the vaccine composition comprises an antigen or antigen composition and an adjuvant composition consisting of an oil in water emulsion comprising 0.5 - 10 mg metabolisable oil, 0.5 – 11 mg tocol and 0.1 – 4 mg emulsifying agent, per human dose.

In a further embodiment there is provided the use of a vaccine composition in the manufacture of a medicament for use in prophylactic therapy or therapy of a condition or disease wherein the vaccine composition comprises an antigen or antigen composition and an adjuvant composition consisting of an oil in water emulsion comprising 0.5-10 mg metabolisable oil, 0.5– 11 mg tocol and 0.1 – 4 mg emulsifying agent, per human dose.

The invention will be further described by reference to the following, non-limiting, examples:

Example I describes immunological read-out methods used in mice, ferrets, pigs and human studies.

Example II describes the preparation of the oil in water emulsion and adjuvant formulations used in the studies exemplified.

Example III shows a clinical trial in an adult population aged 18-59 years with a vaccine containing a split influenza antigen preparation and various doses of AS03 adjuvant

Example IV shows a preclinical evaluation of adjuvanted and non-adjuvanted split influenza vaccines (comprising various doses of AS03 adjuvant) in primed BALB/c mice

Example V shows a preclinical evaluation of adjuvanted and non-adjuvanted split influenza vaccines (comprising various doses of AS03 adjuvant) in primed C57Bl/6 mice

Example VI shows a preclinical evaluation of adjuvanted and non-adjuvanted split influenza vaccines (comprising various doses of AS03 adjuvant and low dose antigen) in primed C57Bl/6 mice

Example VII shows a preclinical evaluation of adjuvanted and non-adjuvanted split H5N1 vaccines (comprising various doses of AS03 adjuvant and antigen) in naïve C57Bl/6 mice

Example VIII shows a preclinical evaluation of adjuvanted and non-adjuvanted influenza vaccines in primed Large White pigs

Example I – Immunological Read-out Methods

I.1. Mice methods

I.1.1. Hemagglutination Inhibition Test

Test principle (classical procedure).

Anti-Hemagglutinin antibody titers to the three (seasonal) influenza virus strains are determined using the hemagglutination inhibition test (HI). The principle of the HI test is based on the ability of specific anti-Influenza antibodies to inhibit hemagglutination of red blood cells (RBC) by influenza virus hemagglutinin (HA). Heat inactivated sera are treated by Kaolin and RBC to remove non-specific inhibitors. After pretreatment, two-fold dilutions of sera are incubated with 4 hemagglutination units of each influenza strain. Red blood cells are then added and the inhibition of agglutination is scored. The titers are expressed as the reciprocal of the highest dilution of serum that completely inhibited hemagglutination. As the first dilution of sera is 1:20, an undetectable level is scored as a titer equal to 10.

Adaptation for H5N1 (specific description of HI using Horse erythrocytes):

As the classical HI assay for determining anti-HA antibodies was documented to not well function for the H5N1 strain, and adapted protocol using horse RBC was used.

Erythrocytes of horses are used for the H5N1 Pandemic strains. 0.5 % (end concentration) horse red blood cell suspension in phosphate buffer containing 0.5 % BSA (bovine serum albumin, end concentration). This suspension is prepared every day by washing red blood cell with the same phosphate buffer and a subsequent centrifugation step (10 min, 2000 rpm). This washing step has to be repeated once. After the addition of the horse red blood cells to the reaction mix of sera and virus suspension; the plates have to be incubated at room temperature (RT, 20°C +/- 2°C) for two hours due to the low sedimentation rate of the horse red blood cells.

Statistical analysis

Statistical analysis were performed on post vaccination HI titers using UNISTAT. The protocol applied for analysis of variance can be briefly described as follow:

- Log transformation of data

- Shapiro-Wilk test on each population (group) in order to verify the normality of groups distribution
- Cochran test in order to verify the homogenicity of variance between the different populations (groups)
- Analysis of variance on selected data.
- Test for interaction of two-way ANOVA
- Tukey-HSD Test for multiple comparisons

I.1.2. Intracellular cytokine staining

This technique allows a quantification of antigen specific T lymphocytes on the basis of cytokine production: effector T cells and/or effector-memory T cells produce IFN-γ and/or central memory T cells produce IL-2. PBMCs are harvested at day 7 post-immunization.

Lymphoid cells are re-stimulated in vitro in the presence of secretion inhibitor (Brefeldine). These cells are then processed by conventional immunofluorescent procedure using fluorescent antibodies (CD4, CD8, IFN-γ and IL-2). Results are expressed as a frequency of cytokine positive cell within CD4/CD8 T cells. Intracellular staining of cytokines of T cells was performed on PBMC 7 days after the second immunization. Blood was collected from mice and pooled in heparinated medium RPMI+ Add. For blood, RPMI + Add-diluted PBL suspensions were layered onto a Lympholyte–Mammal gradient according to the recommended protocol (centrifuge 20 min at 2500 rpm and R.T.). The mononuclear cells at the interface were removed, washed 2x in RPMI + Add and PBMCs suspensions were adjusted to 2 x 10^6 cells/ml in RPMI 5% fetal calf serum.

In vitro antigen stimulation of PBMCs was carried out at a final concentration of 1 x 10^7 cells/ml (tube FACS) with Whole FI (1µgHA/strain) and then incubated 2 hrs at 37°C with the addition of anti-CD28 and anti-CD49d (1 µg/ml for both).

Following the antigen restimulation step, PBMC are incubated overnight at 37°C in presence of Brefeldin (1µg/ml) at 37°C to inhibit cytokine secretion.

IFN-γ /IL-2/CD4/CD8 staining was performed as follows: Cell suspensions were washed, resuspended in 50µl of PBS 1% FCS containing 2% Fc blocking reagent (1/50; 2.4G2). After 10 min incubation at 4°C, 50 µl of a mixture of anti-CD4-PE (2/50) and anti-CD8 perCp (3/50) was added and incubated 30 min at 4°C. After a washing in PBS 1% FCS, cells were permeabilized by resuspending in 200 µl of Cytofix-Cytoperm (Kit BD) and incubated 20 min at 4°C. Cells were then washed with Perm Wash (Kit BD) and resuspended with 50 µl of a mix of anti- IFN-γ APC (1/50) + anti-IL-2 FITC (1/50) diluted in Perm Wash. After an incubation min 2 h max overnight at 4°C, cells were washed with Perm Wash and resuspended in PBS 1% FCS + 1% paraformaldéhyde. Sample analysis was performed by FACS. Live cells were gated (FSC/SSC) and acquisition was performed on ~ 20,000 events (lymphocytes) or 35,000 events on CD4+T cells. The percentages of IFN-γ + or IL2+ were calculated on CD4+ and CD8+ gated populations.

I.1.3. Anti-H5N1 ELISA.

Quantitation of anti-H5N1 Ig, IgG1 and IgG2b antibody titers was performed by ELISA using split H5N1 as coating. Virus and antibody solutions were used at 100 µl per well. Split virus H5N1 was diluted at a final concentration of 1 µg/ml in PBS and was adsorbed overnight at 4°C to the wells of 96 wells microtiter plates (Maxisorb Immunoplate Nunc 439454). The plates were then incubated for 1 hour at 37°C with 200 µl per well of PBS containing 1% BSA and 0.1% Tween 20 (saturation buffer). Twelve two-fold dilutions of sera in saturation buffer were added to the H5N1-coated plates and incubated for 1h30 at 37°C. The plates were washed four times with PBS 0.1% Tween 20. Biotinilated-conjugated anti-mouse Ig (Prozan-E0413) diluted 1/500 or Biotinilated-conjugated anti-mouse IgG1(Imtech 1070-08) ,or a biotynilated anti-mouse IgG2b (Imtech 1090-08) dimuated 1/4000 in PBS 1% BSA 0.1% Tween 20 was added to each well and incubated for 1.30 hour at 37°C; after a washing step, plates were incubated 30 min with a Streptavidine-Biotine-Preoxidase conjugated (Prozan P0397) diluated 1/10000 in PBS 1% BSA Tween 20.

For the colorimetric revelation, plates were incubated 20 min at 22°C with a solution of o-phenyldiamine (Sigma P4664) 0.04% H2O2 0.03% in 0.1 M citrate buffer pH 4.2. The reaction was stopped with H_2SO_4 2N and micoplates were read at 490-630 nm.

I.2. Ferrets methods

I.2.1. Hemagglutination Inhibition Test (HI)

Test procedure.

Anti-Hemagglutinin antibody titers to the three influenza virus strains were determined using the hemagglutination inhibition test (HI). The principle of the HI test is based on the ability of specific anti-Influenza antibodies to inhibit hemagglutination of chicken red blood cells (RBC) by influenza virus hemagglutinin (HA). Sera were first treated with a 25% neuraminidase solution (RDE) and were heat-inactivated to remove non-specific inhibitors. After pre-treatment, two-fold dilutions of sera were incubated with 4 hemagglutination units of each influenza strain. Chicken red blood cells were then added and the inhibition of agglutination was scored. The titers were expressed as the reciprocal of the highest dilution of serum that completely inhibited hemagglutination. As the first dilution of sera was 1:10, an undetectable level was scored as a titer equal to 5.

Statistical analysis.

Statistical analysis were performed on HI titers (Day 41, before challenge) using UNISTAT. The protocol applied for analysis of variance can be briefly described as followed:

- Log transformation of data.
- Shapiro-wilk test on each population (group) in ordetr to verify the normality of groups distribution.
- Cochran test in order to verify the homogenicity of variance between the different populations (groups).
- Test for interaction of one-way ANOVA.
- Tuckey-HSD Test for multiple comparisons.

I.2.2. Body temperature monitoring

Individual temperatures were monitored during the challenge period with the transmitters and by the telemetry recording. All implants were checked and refurbished and a new calibration was performed by DSI (Data Sciences International, Centaurusweg 123, 5015 TC Tilburg, The Netherlands) before placement in the intraperitoneal cavity. All animals were individually housed in single cage during these measurements.

Temperatures were recorded every 15 minutes 4 days before challenge until 7 days Post-challenge.

I.2.3. Nasal washes

The nasal washes were performed by administration of 5 ml of PBS in both nostrils in awoke animals. The inoculum was collected in a Petri dish and placed into sample containers on dry ice.

Viral titration in nasal washes

All nasal samples were first sterile filtered through Spin X filters (Costar) to remove any bacterial contamination. 50 µl of serial ten-fold dilutions of nasal washes were transferred to microtiter plates containing 50 µl of medium (10 wells/dilution). 100µl of MDCK cells (2.4×10^5 cells/ml) were then added to each well and incubated at 35°C for 5-7days.

After 5-7 days of incubation, the culture medium is gently removed and 100 µl of a 1/20 WST-1 containing medium is added and incubated for another 18 hrs.

The intensity of the yellow formazan dye produced upon reduction of WST-1 by viable cells is proportional to the number of viable cells present in the well at the end of the viral titration assay and is quantified by measuring the absorbance of each well at the appropriate wavelength (450 nanometers). The cut-off is defined as the OD average of uninfected control cells – 0.3 OD (0.3 OD correspond to +/- 3 StDev of OD of uninfected control cells). A positive score is defined when OD is < cut-off and in contrast a negative score is defined when OD is > cut-off. Viral shedding titers were determined by "Reed and Muench" and expressed as Log TCID50/ml.

I.3. Pig methods

I.3.1. Hemagglutination Inhibition Test (HI)

Test procedure.

Anti-Hemagglutinin antibody titers to the three influenza virus strains were determined using the hemagglutination inhibition test (HI). The principle of the HI test is based on the ability of specific anti-Influenza antibodies to inhibit hemagglutination of chicken red blood cells (RBC) by influenza virus hemagglutinin (HA). Sera were first treated with a 25% neuraminidase solution (RDE) and were heat-inactivated to remove non-specific inhibitors. After pre-treatment, two-fold dilutions of sera were incubated with 4 hemagglutination units of each influenza strain. Chicken red blood cells were then added and the inhibition of agglutination was scored. The titers were expressed as the reciprocal of the highest dilution of serum that completely inhibited hemagglutination. As the first dilution of sera was 1:10, an undetectable level was scored as a titer equal to 5.

Statistical analysis.

Statistical analysis were performed on HI titers (Day 41, before challenge) using UNISTAT. The protocol applied for analysis of variance can be briefly described as followed:

- Log transformation of data.
- Shapiro-wilk test on each population (group) in ordetr to verify the normality of groups distribution.
- Cochran test in order to verify the homogenicity of variance between the different populations (groups).
- Test for interaction of one-way ANOVA.
- Tuckey-HSD Test for multiple comparisons.

I.4. Assays for assessing the immune response in humans

I.4.1. Hemagglutination Inhibition Assay

The immune response was determined by measuring HI antibodies using the method described by the WHO Collaborating Centre for influenza, Centres for Disease Control, Atlanta, USA (1991).

Antibody titre measurements were conducted on thawed frozen serum samples with a standardised and comprehensively validated micromethod using 4 hemagglutination-inhibiting units (4 HIU) of the appropriate antigens and a 0.5% fowl erythrocyte suspension. Non-specific serum inhibitors were removed by heat treatment and receptor-destroying enzyme.

The sera obtained were evaluated for HI antibody levels. Starting with an initial dilution of 1:10, a dilution series (by a factor of 2) was prepared up to an end dilution of 1:20480.

The titration end-point was taken as the highest dilution step that showed complete inhibition (100%) of hemagglutination. All assays were performed in duplicate.

I.4.2. Neuraminidase Inhibition Assay

The assay was performed in fetuin-coated microtitre plates. A 2-fold dilution series of the antiserum was prepared and mixed with a standardised amount of influenza A H3N2, H1N1 or influenza B virus. The test was based on the biological activity of the neuraminidase which enzymatically releases neuraminic acid from fetuin. After cleavage of the terminal neuraminic acid ß-D-glactose-N-acetyl-galactosamin was unmasked. Horseradish peroxidase (HRP)-labelled peanut agglutinin from Arachis hypogaea, which binds specifically to the galactose structures, was added to the wells. The amount of bound agglutinin can be detected and quantified in a substrate reaction with tetra-methylbenzidine (TMB) The highest antibody dilution that still inhibits the viral neuraminidase activity by at least 50% was indicated is the NI titre.

I.4.3. Neutralising Antibody Assay

Neutralising antibody measurements were conducted on thawed frozen serum samples. Virus neutralisation by antibodies contained in the serum was determined in a microneutralization assay. The sera were used without further treatment in the assay. Each serum was tested in triplicate. A standardised amount of virus was mixed with serial dilutions of serum and incubated to allow binding of the antibodies to the virus. A cell suspension, containing a defined amount of MDCK cells was then added to the mixture of virus and antiserum and incubated at 33°C. After the incubation period, virus replication was visualised by hemagglutination of chicken red blood cells. The 50% neutralisation titre of a serum was calculated by the method of Reed and Muench.

I.4.4. Cell-mediated Immunity was evaluated by Cytokine Flow Cytometry (CFC)

Peripheral blood antigen-specific CD4 and CD8 T cells can be restimulated *in vitro* to produce IL-2, CD40L, TNF-alpha and IFN if incubated with their corresponding antigen. Consequently, antigen-specific CD4 and CD8 T cells can be enumerated by flow cytometry following conventional immunofluorescence labelling of cellular phenotype as well as intracellular cytokines production. In the present study, Influenza vaccine antigen as well as peptides derived from specific influenza protein were used as antigen to restimulate Influenza-specific T cells. Results were expressed as a frequency of cytokine(s)-positive CD4 or CD8 T cell within the CD4 or CD8 T cell sub-population.

I.4.5. Statistical Methods

I.4.5.1. Primary endpoints

• Percentage, intensity and relationship to vaccination of solicited local and general signs and symptoms during a 7 day follow-up period (i.e. day of vaccination and 6 subsequent days) after vaccination and overall.

• Percentage, intensity and relationship to vaccination of unsolicited local and general signs and symptoms during a 21 day follow-up period (i.e. day of vaccination and 20 subsequent days) after vaccination and overall.

• Occurrence of serious adverse events during the entire study.

I.4.5.2. Secondary endpoints

For the humoral immune response:

Observed variables:

• At days 0 and 21: serum hemagglutination-inhibition (HI) and NI antibody titres, tested separately against each of the three influenza virus strains represented in the vaccine (anti-H1N1, anti-H3N2 & anti-B-antibodies).

• At days 0 and 21: neutralising antibody titres, tested separately against each of the three influenza virus strains represented in the vaccine

Derived variables (with 95% confidence intervals):

• Geometric mean titres (GMTs) of serum HI antibodies with 95% confidence intervals (95% CI) pre and post-vaccination

• Seroconversion rates* with 95% CI at day 21

• Conversion factors** with 95% CI at day 21

• Seroprotection rates*** with 95% CI at day 21

• Serum NI antibody GMTs' (with 95% confidence intervals) at all timepoints.

* Seroconversion rate defined as the percentage of vaccinees who have at least a 4-fold increase in serum HI titres on day 21 compared to day 0, for each vaccine strain.

**Conversion factor defined as the fold increase in serum HI GMTs on day 21 compared to day 0, for each vaccine strain.

***Protection rate defined as the percentage of vaccinees with a serum HI titre =40 after vaccination (for each vaccine strain) that usually is accepted as indicating protection.

It should be understood, that for some of the clinical trials, reactogenicity/safety may be secondary endpoints, and immunogenicity may be the primary endpoint.

For the cell mediated immune (CMI) response

Observed variable

At days 0 and 21: frequency of cytokine-positive CD4/CD8 cells per 10^6 in different tests. Each test quantifies the response of CD4/CD8 T cell to:

- Peptide Influenza (pf) antigen (the precise nature and origin of these antigens needs to be given/explained
- Split Influenza (sf) antigen
- Whole Influenza (wf) antigen.

Derived variables:

- cells producing at least two different cytokines (CD40L, IL-2, IFNγ, TNFα)
- cells producing at least CD40L and another cytokine (IL-2, TNFα, IFNγ)
- cells producing at least IL-2 and another cytokine (CD40L, TNFα, IFNγ)
- cells producing at least IFNγ and another cytokine (IL-2, TNFα, CD40L)
- cells producing at least TNFα and another cytokine (IL-2, CD40L, IFNγ)

I.3.5.3. Analysis of immunogenicity

The immunogenicity analysis was based on the total vaccinated cohort. For each treatment group, the following parameters (with 95% confidence intervals) were calculated:

- Geometric mean titres (GMTs) of HI and NI antibody titres at days 0 and 21
- Geometric mean titres (GMTs) of neutralising antibody titres at days 0 and 21.
- Conversion factors at day 21.
- Seroconversion rates (SC) at day 21 defined as the percentage of vaccinees that have at least a 4-fold increase in serum HI titres on day 21 compared to day 0.
- Protection rates at day 21 defined as the percentage of vaccinees with a serum HI titre =1:40.
- The frequency of CD4/CD8 T-lymphocytes secreting in response was summarised (descriptive statistics) for each vaccination group, at each timepoint (Day 0, Day 21) and for each antigen (Peptide influenza (pf), split influenza (sf) and whole influenza (wf)).
- Descriptive statistics in individual difference between timepoint (Post-Pre) responses fore each vaccination group and each antigen (pf, sf, and wf) at each 5 different tests.
- A non-parametric test (Kruskall-Wallis test) was used to compare the location

differences between the 3 groups and the statistical p-value was calculated for each antigen at each 5 different tests. All significance tests were two-tailed. P-values less than or equal to 0.05 were considered as statistically significant.

Example II – Preparation of the oil in water emulsion and adjuvant formulations

Unless otherwise stated, the oil/water emulsion used in the subsequent examples is composed an organic phase made of 2 oils (alpha-tocopherol and squalene), and an aqueous phase of PBS containing Tween 80 as emulsifying agent. Unless otherwise stated, the oil in water emulsion adjuvant formulations used in the subsequent examples were made comprising the following oil in water emulsion component (final concentrations given): 2.5% squalene (v/v), 2.5% alpha-tocopherol (v/v), 0.9% polyoxyethylene sorbitan monooleate (v/v) (Tween 80), see WO 95/17210. This emulsion, termed AS03 in the subsequent examples, was prepared as followed as a two-fold concentrate.

II.1. Preparation of emulsion SB62

This method was used in the studies reported in the clinical and pre-clinical examples sections. The preparation of the SB62 emulsion is made by mixing under strong agitation of an oil phase composed of hydrophobic components (DL-α-tocopherol and squalene) and an aqueous phase containing the water soluble components (the anionic detergent Tween 80 and PBS mod (modified), pH 6.8). While stirring, the oil phase (1/10 total volume) is transferred to the aqueous phase (9/10 total volume), and the mixture is stirred for 15 minutes at room temperature. The resulting mixture then subjected to shear, impact and cavitation forces in the interaction chamber of a microfluidizer (15000 PSI – 8 cycles, or 3 cycles in the adjuvant used in the clinical trial reported in Example III) to produce submicron droplets (distribution between 100 and 200 nm). The resulting pH is between 6.8 ± 0.1. The SB62 emulsion is then sterilised by filtration through a 0.22 µm membrane and the sterile bulk emulsion is stored refrigerated in Cupac containers at 2 to 8°C. Sterile inert gas (nitrogen or argon) is flushed into the dead volume of the SB62 emulsion final bulk container for at least 15 seconds.

The final composition of the SB62 emulsion is as follows :
Tween 80: 1.8 % (v/v) 19.4 mg/ml; Squalene: 5 % (v/v) 42.8 mg/ml; α-tocopherol: 5 % (v/v) 47.5 mg/ml; PBS-mod: NaCl 121 mM, KCl 2.38 mM, Na2HPO4 7.14 mM, KH2PO4 1.3 mM; pH 6.8 ± 0.1.

Example III – Clinical trial in an adult population aged 18-59 years with a vaccine containing a split influenza antigen preparation and various doses of AS03 adjuvant (Flu-LD-004)

III.1. Introduction

A phase II, controlled, randomized, single blind study was conducted in an adult population aged 18-59 years old in 2006 in order to evaluate the immunogenicity, safety and reactogenicity of the GlaxoSmithKline Biologicals low dose influenza candidate vaccine (i.e. containing 5µg HA per strain) with two doses of AS03 adjuvant.
The humoral immune response (i.e. anti-hemagglutinin) was measured 21 days after intramuscular administration of one dose of an AS03 adjuvanted vaccine. Fluarix™ was used as reference.

III.2. Study design

Three groups of subjects in parallel received the following vaccine intramuscularly:

- one group of 100 subjects receiving one injection of the low dose split virus influenza vaccine containing 5µg HA adjuvanted with AS03 (FluLD1/1)
- one group of 100 subjects receiving one injection of the low dose split virus influenza vaccine containing 5µg HA adjuvanted with a half dose of AS03 (AD03 ½) (FluLD1/2)
- one group of 100 subjects receiving one dose of Fluarix™ (Fluarix)

Schedule: one IM injection of influenza vaccine at day 0, study site visits at day 0 and day 21 with a blood sample collection (HI antibody determination) and an additional phone contact at day 30 (study conclusion).

The standard trivalent split influenza vaccine - Fluarix™ used in this study, is a commercial vaccine from the year 2006 developed and manufactured by GlaxoSmithKline Biologicals.

III.3. Study Objectives

III.3.1. Primary objective

- To evaluate the humoral immune response induced by the study vaccines in term of anti-haemagglutinin antibody titers:

Observed variables at days 0 and 21: serum Heamagglutination-inhibition antibody titers.
Derived variables (with 95% confidence intervals):

- Geometric mean titers (GMTs) of serum antibodies at days 0 and 21
- Seroconversion rates* at day 21
- Conversion factors** at day 21
- Protection rates*** at days 0 and 21

* Seroconversion rate for Haemagglutinin antibody response is defined as the percentage of vaccinees who have either a prevaccination titer < 1:10 and a post-vaccination titer ≥ 1:40 or a prevaccination titer ≥ 1:10 and at least a fourfold increase in post-vaccination titer

**Conversion factor defined as the fold increase in serum HI GMTs post-vaccination compared to day 0;

***Protection rate defined as the percentage of vaccinees with a serum HI titer ≥40 after vaccination that usually is accepted as indicating protection.

III.3.2. Secondary objective

- To evaluate the safety and reactogenicity of the study vaccines in term of solicited local and general adverse events, unsolicited adverse events and serious adverse events:

1. Occurence, intensity and relationship to vaccination of solicited local and general signs and symptoms during a 7-day follow-up period (i.e. day of vaccination and 6 subsequent days) after each vaccination in each group.
2. Occurence, intensity and relationship to vaccination of unsolicited local and general signs and symptoms during a 30-day follow-up period (i.e. day of vaccination and 29 subsequent days) after the vaccination in each group.
3. Occurrence and relationship of serious adverse events during the entire study period in each group.

III.4. Vaccine composition and administration

III.4.1. Vaccine preparation

The non-adjuvanted influenza vaccine is a trivalent split virion, inactivated influenza vaccine consisting of three monovalent viral antigen bulks (prepared from respectively

influenza strains A/H1N1, A/H3N2 and B). The antigens present in this vaccine are the same as in the licensed *Fluarix*™ vaccine which is available on the market as *Fluarix* ™ (*α-Rix*®) since 1992 and contain 15 μg HA/strain per dose. The influenza strains included in the FluLD clinical lots are the strains that were chosen for the 2006/2007 Northern Hemisphere:

- A/New Caledonia/20/99 (H1N1)-like strain: A/New Caledonia/20/99 (H1N1) IVR-116
- A/Wisconsin/67/2005 (H3N2)-like strain: A/Wisconsin/67/2005 (H3N2) NYMCX-161
- B/Malaysia/2506/2004.

The antigens are derived from egg-grown viruses. Splitting is carried out with sodium deoxycholate prior to the inactivation step, which is performed through the subsequent action of sodium deoxycholate and formaldehyde.

The AS03 adjuvanted low dose influenza (FluLD) vaccine (clinical lots) is based on the commercially available *Fluarix*™ vaccine (prepared from respectively influenza strains A/H1N1, A/H3N2 and B), but with a lower antigen content and adjuvanted with GSK adjuvant system AS03. AS03 consists of an oil-in-water emulsion (SB62) that contains two biodegradable oils, squalene and α-tocopherol (Vitamin E), and a surfactant, polysorbate 80 (Tween 80). Influenza antigens are incorporated in the aqueous phase of the adjuvant system by simple mixing with the emulsion. Two formulations have been tested, differing by the amount of adjuvant introduced with the Flu antigens in the vaccine lot. The adjuvanted vaccines contain 5 μg haemagglutinin (HA) of each influenza virus strain per dose, combined with a full dose (AS03) or half a dose (AS03 ½) of the adjuvant system AS03. The excipients are the following: polysorbate 80 (Tween 80), octoxynol 10 (Triton X-100), alpha-tocopheryl hydrogen succinate, sodium chloride, disodium hydrogen phosphate, potassium dihydrogen phosphate, potassium chloride, water for injection.
The AS03 adjuvanted low dose influenza vaccines (FluLD, full or half dose of AS03) are preservative-free vaccines. However, they contain trace amounts of thiomersal (< 1.25 μg of Hg per dose) from the early stages of the manufacturing process. They are both presented as monodose vaccines in glass (Type I) pre-filled syringes at a volume of 0.5 ml/dose.

III.4.1.1. Composition of AS03 adjuvanted influenza vaccine

One dose of FluLD (full or half dose of AS03) corresponds to 0.5 ml. The composition is provided in Table 3. The HA content per dose is 5 μg for both formulations, the sole difference being the amount of AS03 present in the final containers.

Table 3 Composition of AS03 adjuvanted low dose influenza vaccine (full and half dose of AS03)

Component	Quantity per dose (0.5 ml)
Inactivated split virions - A/New Caledonia/20/99 (H1N1) IVR-116 - A/Wisconsin/67/2005 (H3N2) NYMCX-161 - B/Malaysia/2506/2004	 5 µg HA 5 µg HA 5 µg HA
Adjuvant (Full Dose / HalfDose) - SB62 emulsion (Total Volume) • squalene • DL-α-tocopherol • Polysorbate 80 (Tween 80)	0.250 mL 10.70 mg / 5.35 mg 11.88 mg / 5.94 mg 4.85 mg / 2.425 mg
Polysorbate 80 (Tween 80)	0.122 mg
Octoxynol 10 (Triton X-100)	0.0283 mg
αTocopheryl hydrogen succinate	0.01665 mg
Sodium chloride	4 mg
Disodium phosphate	0.575 mg
Potassium dihydrogen phosphate	0.100 mg
Potassium chloride	0.101mg
Water for injection	ad 0.50 ml

Abbreviations: HA = Haemagglutinin.

The total content in Polysorbate 80 corresponds to 4.972 mg per dose when AS03 full dose is used, and 2.547 mg per dose when AS03 half dose is used.

III.4.1.2. Production of split inactivated influenza antigen preparation

The influenza antigens are identical to those included in *Fluarix*™ (Influenza Virus Vaccine). The monovalent bulks consist of purified inactivated split viruses that are prepared from working seeds of the three strains of influenza virus, type A (H1N1 and H3N2) and type B, which are grown individually in embryonated hens' eggs. These working seeds are derived from strains that are received from a WHO collaborating center following the annual WHO recommendations. For the process for preparing the antigens reference is, by way of illustration, given to WO 02/097072. The volumes of the three monovalent bulks are based on the HA content measured in each monovalent bulk prior to the formulation and on the target manufacturing volume.

A 10-times concentrated phosphate buffered saline (pH 7.4 when 1 time concentrated) and a pre-mixture of Tween 80 and α-tocopheryl hydrogen succinate are diluted in water for injection, followed by stirring during 5-30 minutes at room temperature.

The three concentrated monovalent bulks are then successively diluted in the resulting phosphate buffered saline / Tween 80 - α-tocopheryl hydrogen succinate solution to a concentration of

20 µg HA of each A monovalent bulk (H1N1, H3N2)

23.32 µg HA of B monovalent bulk

per mL of intermediate trivalent bulk (5 µg HA of each A monovalent bulk and 5.83 µg HA of B / 500 µl trivalent final bulk).

Between the additions of each monovalent bulk, the mixture is stirred for 10 - 30 minutes at room temperature and for 15 - 30 minutes after addition of the last monovalent bulk. This intermediate trivalent bulk also referred to as "pre-pool" can be held at +2 - +8°C or further processed to the final formulation step on the same day. The final volume of pre-pool is 250 µl per dose.

III.4.1.3. Preparation of the vaccine compositions with AS03 adjuvant

Adjuvanted vaccine: LD AS03 1/1 (Table 4)

PBS mod 10 fold concentrated (pH 7.4 when one fold concentrated; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4) as well as a mixture containing Tween80, Triton X-100 and VES (quantities taking into account the detergent present in the strains) are added to water for injection. After 5 to 30 minutes stirring, 20 µg HA per ml of each strain H1N1 and H3N2 and 23.32µg HA per ml of B strain are added with 10 to 30 minutes stirring between each addition. After 15 to 30 minutes stirring, a small volume of the so called "intermediate bulk" are discarded for analysis and stored between +2 and +8°C. The intermediate bulk is in PBS mod 1 fold concentrated. The target's detergents concentration are 488µg Tween 80 per ml, 73.6µg Triton X-100 per ml and 66.6µg VES per ml.

The final formulation is then prepared: an equal volume of SB62 (see preparation in Example II) is added to each 250 µl of pre-pool intermediate bulk and mixed during 30 to 60 minutes at room temperature. pH is checked to range between 6.8 and 7.5. Formulation is flushed with nitrogen and then stored between +2 and 8°C prior to filling.

Table 4 AS03 adjuvanted low dose vaccine

Component	Concentration	Volume (ml)
Step 1: Prepool		
A/New Caledonia monovalent bulk	104µg/ml	302.88
A/ Wisconsin monovalent bulk	85µg/ml	370.59
B/ Malaysia monovalent bulk	110 µg/ml	333.90
PBS mod(1)	See legend	56.76
Tween 80	48000 µg/ml	5.24
Triton X-100		Residual from H3N2 strain
α-tocopheryl hydrogen succinate	26480 µg/ml	1.2
Filtrated water		504.43
Total volume = 1575(ml) *75ml of prepool samples are retrieved for testing* Remaining prepool volume = 1500(ml)		
Step 2: added to prepool		
Emulsion SB62		1500
Total volume of final bulk = 3000(ml)		

(1): The buffer final bulk composition is: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4

Adjuvanted vaccine: LD AS03 1/2 (Table 5)

PBS mod 10 fold concentrated (pH 7.4 when one fold concentrated – see composition above) as well as a mixture containing Tween 80, Triton X-100 and VES (quantities taking into account the detergent present in the strains) are added to water for injection. After 5 to 30 minutes stirring, 20 µg HA per ml of each strain H1N1 and H3N2 and 23.32µg HA per ml of B strain are added with 10 to 30 minutes stirring between each addition. After 15 to 30 minutes stirring, a small volume of the so called "intermediate bulk" are discarded for analysis and stored between +2 and +8°C. PBS mod is 1 fold concentrated in the intermediate bulk. The target's detergents concentration are 488µg Tween 80 per ml, 73.6µg Triton X-100 per ml and 66.6µg VES per ml

Final formulation is then prepared: SB62 is first diluted with the PBS mod buffer and stirred for 15 – 30 minutes at RT. An equal volume of this diluted SB62 is then added to each 250 µl of pre-pool of intermediate bulk. After 30 to 60 minutes stirring at RT, pH is checked to range between 6.8 and 7.5. Formulation is flushed with nitrogen and then stored between +2 and 8°C prior to filling.

The final volume of both formulation is 500 µl per dose and the final HA concentration is 10 µg of each A monovalent bulk and 11.66 µg of B monovalent bulk per ml of trivalent final bulk. Final Tween 80, Triton X-100 (residual from H3N2 monobulk manufacturing) and α -tocopheryl hydrogen succinate (α-tocopheryl hydrogen succinate is an ester form

of RRR (D isomer)-α-tocopherol) target concentrations are 244 μg/ml, 58.6 μg/ml and 33.3 μg/ml, respectively.

Table 5 AS03 adjuvanted low dose vaccine (half-dose of adjuvant)

Component	Concentration	Volume (ml)
Step 1: Prepool		
Step 1: Prepool		
A/New Caledonia monovalent bulk	104μg/ml	300.96
A/ Wisconsin monovalent bulk	85μg/ml	368.24
B/ Malaysia monovalent bulk	110 μg/ml	331.78
PBS mod(1)	See legend	56.4
Tween 80	48000 μg/ml	5.2
Triton X-100		Residual from H3N2 strain
α-tocopheryl hydrogen succinate	26480 μg/ml	1.2
Filtrated water		501.22
Total volume = 1565(ml) *65ml of prepool samples are retrieved for testing* Remaining prepool volume = 1500(ml)		
Step 2: added to prepool		
Emulsion SB62		750
PBS mod(1)	See legend	75
Filtrated water		675
Total volume of final bulk = 3000(ml)		

(1): The buffer final bulk composition is: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4

III.4.2. Vaccine administration

The vaccine is filled into 1.25-ml sterile Type I (Ph. Eur.) glass syringes. Each syringe is filled to a target of 0.57 ml (range: 0.54-0.60 ml). The vaccines were administered intramuscularly in the deltoid region of the non-dominant arm. All vaccines were presented as pre-filled syringes (0.5 ml). In order to ensure proper IM injection of the vaccine, a needle of at least 25G and at least 2.5 cm in length was used.

III.5 Study population results

A total of 300 subjects were enrolled in this study: 100 subjects in each of the 3 groups. The mean age of the total vaccinated cohort at the time of vaccination was 36.7 years with a standard deviation of 13.67 years. The mean age and gender distribution of the subjects across the 3 vaccine groups was similar.

III.6 Immunogenicity results

Analysis of immunogenicity was performed on the ATP cohort for immunogenicity (297 subjects).

Humoral immune response

In order to evaluate the humoral immune response induced by the low dose influenza candidate vaccine adjuvanted with AS03, the following parameters (with 95% confidence intervals) were calculated for each treatment group:

- Geometric mean titres (GMTs) of HI antibody titres at days 0 and 21;
- Seroconversion rates (SC) at days 21;
- Conversion factors at day 21;
- Protection rates at day 0 and 21.

III.6.1 HI Geometric mean titres (GMT)

The GMTs for HI antibodies with 95% CI are shown in Table 10 and Figure 1. Adjusted GMT ratios between groups are shown in Table 11.

Pre-vaccination GMTs of HI antibodies for all 3 vaccine strains were within the same range in the 3 treatment groups. The observed GMTs at day 21 for adjuvanted groups tends to be higher than Fluarix group for all 3 strains with a statistical difference (no overlapping of 95%CIs and adjusted GMT ratio did not contain the value 1) between FluLD1/1 and Fluarix for the A/Wisconsin vaccine strain. A statistical difference (adjusted GMT ratio did not contain the value 1) was observed also between FluLD1/2 and Fluarix for the B/Malaysia vaccine strain.

Table 10 - Seropositivity rates and Geometric mean titers (GMTs) for anti-HA antibody at day 0 and 21 (ATP cohort for immunogenicity)

Antibody	Group	Timing	N	≥ 10 1/DIL				GMT			Min	Max
				n	%	95% CI		1/DL	95% CI			
						LL	UL		LL	UL		
A/New Caledonia	FluLD1/1	PRE	99	80	80.8	71.7	88.0	31.9	23.5	43.4	<10.0	2560.0
		PI(D21)	99	99	100	96.3	100	475.4	352.2	641.6	20.0	7240.0
	FluLD1/2	PRE	99	80	80.8	71.7	88.0	36.1	26.9	48.5	<10.0	3620.0
		PI(D21)	99	98	99.0	94.5	100	399.0	294.7	540.2	<10.0	7240.0
	Fluarix	PRE	98	85	86.7	78.4	92.7	26.1	20.5	33.2	<10.0	1280.0
		PI(D21)	98	98	100	96.3	100	380.6	274.2	528.4	10.0	7240.0
A/Wisconsin	FluLD1/1	PRE	99	61	61.6	51.3	71.2	16.8	13.1	21.5	<10.0	453.0
		PI(D21)	99	99	100	96.3	100	276.2	223.5	341.3	28.0	5120.0
	FluLD1/2	PRE	99	66	66.7	56.5	75.8	19.9	15.2	25.9	<10.0	640.0
		PI(D21)	99	99	100	96.3	100	241.9	192.9	303.4	20.0	5120.0
	Fluarix	PRE	98	58	59.2	48.8	69.0	14.7	11.6	18.6	<10.0	320.0
		PI(D21)	98	97	99.0	94.4	100	172.3	136.4	217.6	<10.0	5120.0
B/Malaysia	FluLD1/1	PRE	99	72	72.7	62.9	81.2	20.4	15.9	26.1	<10.0	453.0
		PI(D21)	99	99	100	96.3	100	268.6	221.3	326.0	28.0	2560.0
	FluLD1/2	PRE	99	76	76.8	67.2	84.7	22.2	17.6	27.9	<10.0	320.0
		PI(D21)	99	99	100	96.3	100	301.5	246.1	369.4	28.0	3620.0
	Fluarix	PRE	98	76	77.6	68.0	85.4	26.5	20.9	33.6	<10.0	320.0
		PI(D21)	98	97	99.0	94.4	100	219.2	171.4	280.2	<10.0	5120.0

FluLD1/1 = Low dose influenza vaccine (5 ug HA/strain) with full dose of AS03 adjuvant
FluLD1/2 = Low dose influenza vaccine (5 ug HA/strain) with half dose of AS03 adjuvant
Fluarix = Fluarix vaccine
GMT = Geometric Mean antibody Titer
N = Number of subjects with available results
n/% = number/percentage of seropositive subjects (HI titer >= 1:10)
95% CI = 95% confidence interval, LL = Lower Limit, UL = Upper Limit
MIN/MAX = Minimum/Maximum
PRE = Pre-vaccination at day 0
PI (D21) = Post-vaccination at Day 21

TABLE 11 - Adjusted GMT ratios between groups for each vaccine strain at day 21 (ATP cohort for immunogenicity)

Antibody	Group description	N	Adjusted GMT	Group description	N	Adjusted GMT	Adjusted GMT ratio			
							Ratio order	Value	95% CI	
									LL	UL
A/New Caledonia (1/DIL)	FluLD1/1	99	472.4	FluLD1/2	99	385.0	FluLD1/1 /FluLD1/2	1.23	0.80	1.88
	FluLD1/1	99	472.3	Fluarix	98	396.9	FluLD1/1 /Fluarix	1.19	0.78	1.82
	FluLD1/2	99	385.0	Fluarix	98	397.0	FluLD1/2 /Fluarix	0.97	0.63	1.49
A/Wisconsin (1/DIL)	FluLD1/1	99	277.3	FluLD1/2	99	230.0	FluLD1/1 /FluLD1/2	1.21	0.90	1.62
	FluLD1/1	99	277.5	Fluarix	98	180.8	FluLD1/1 /Fluarix	1.54	1.14	2.06
	FluLD1/2	99	230.0	Fluarix	98	180.6	FluLD1/2 /Fluarix	1.27	0.95	1.71
B/Malaysia (1/DIL)	FluLD1/1	99	275.1	FluLD1/2	99	303.4	FluLD1/1 /FluLD1/2	0.91	0.68	1.22
	FluLD1/1	99	275.2	Fluarix	98	212.7	FluLD1/1 /Fluarix	1.29	0.96	1.74
	FluLD1/2	99	303.4	Fluarix	98	212.6	FluLD1/2 /Fluarix	1.43	1.06	1.92

FluLD1/1 = Low dose influenza vaccine (5 ug HA/strain) with full dose of AS03 adjuvant

FluLD1/2 = Low dose influenza vaccine (5 ug HA/strain) with half dose of AS03 adjuvant
Fluarix = Fluarix vaccine
Adjusted GMT = geometric mean antibody titre adjusted for baseline titre
N = Number of subjects with both pre- and post-vaccination results available
95% CI = 95% confidence interval for the adjusted GMT ratio (Ancova model: adjustment for baseline titre - pooled variance with more than 2 groups); LL = lower limit, UL = upper limit

III.6.2 Conversion factors of anti-HI antibody titres, seroprotection rates and seroconversion rates (correlates for protection as established for influenza vaccine in humans)

Results are presented in Table 6 - Figure 2 for seroprotection rates, Table 7 - Figure 3 for seroconversion rates and Table 8 - Figure 4 for conversion factors.

The threshold required by the European Authorities for the **seroprotection rates** (70 %) was reached in all groups (at least 94.9 %). For each vaccine strain, the seroprotection rates at day 21 for the 3 groups were within the same range.

The threshold required by the European Authorities for the **seroconversion rates** (40 %) was reached in all groups (at least 65 %).

For the A/New Caledonia vaccine strain, the SCR at day 21 for the 3 groups were within the same range.

For the A/Wisconsin vaccine strain, the SCR at day 21 for the FluLD1/1 group tended to be higher compared to the Fluarix group. The SCR at day 21 for the FluLD1/2 group was within the same range compared to the Fluarix group.

For the B/Malaysia vaccine strain, the SCR at day 21 for the FluLD1/2 group tended to be higher compared to the Fluarix group. The SCR at day 21 for the FluLD1/1 group was within the same range compared to the Fluarix group.

The threshold required by the European Authorities for the **seroconversion factors** (2.5) was reached in all groups (at least 6.2).

For the A/New Caledonia vaccine strain, the SCF at day 21 for the 3 groups seemed to be within the same range. The observed value for FluLD1/2 group was lower than the observed value for the Fluarix group but could be explained by the higher pre-vaccination seroprotection rate in the FluLD1/2 group.

For the A/Wisconsin vaccine strain, the SCF at day 21 for the FluLD1/1 group tended to be higher compared to the Fluarix group. The SCF at day 21 for the FluLD1/2 group was within the same range compared to Fluarix group.

For the B/Malaysia vaccine strain, the SCF at day 21 for the two adjuvanted groups tended to be higher compared to the Fluarix group.

TABLE 6 - Seroprotection rates (SPR) for HI antibody titer at day 0 and day 21 (ATP cohort for immunogenicity)

Vaccine strain	Group	Timing	N	SPR			
				n	%	95% CI LL	UL
A/New Caledonia	FluLD1/1	PRE	99	41	41.4	31.6	51.8
		PI(D21)	99	95	96.0	90.0	98.9
	FluLD1/2	PRE	99	55	55.6	45.2	65.5
		PI(D21)	99	97	98.0	92.9	99.8
	Fluarix	PRE	98	35	35.7	26.3	46.0
		PI(D21)	98	93	94.9	88.5	98.3
A/Wisconsin	FluLD1/1	PRE	99	32	32.3	23.3	42.5
		PI(D21)	99	97	98.0	92.9	99.8
	FluLD1/2	PRE	99	37	37.4	27.9	47.7
		PI(D21)	99	97	98.0	92.9	99.8
	Fluarix	PRE	98	25	25.5	17.2 * 8.5	35.3 *
		PI(D21)	98	93	94.9		
B/Malaysia	FluLD1/1	PRE	99	31	31.3	22.4	41.4
		PI(D21)	99	97	98.0	92.9	99.8
	FluLD1/2	PRE	99	39	39.4	29.7	49.7
		PI(D21)	99	98	99.0	94.5	100
	Fluarix	PRE	98	44	44.9	34.8	55.3
		PI(D21)	98	94	95.9	89.9	98.9

FluLD1/1 = Low dose influenza vaccine (5 ug HA/strain) with full dose of AS03 adjuvant
FluLD1/2 = Low dose influenza vaccine (5 ug HA/strain) with half dose of AS03 adjuvant
Fluarix = Fluarix vaccine
N = Number of subjects with available results
n/% = Number/percentage of seroprotected subjects (HI titer >= 40 1/DIL)
95% CI = 95% confidence interval, LL = Lower Limit, UL = Upper Limit
PRE = Pre-vaccination at day 0
PI (D1) = Post-vaccination at Day 21
Data source = Appendix table IIIA

TABLE 7 - Seroconversion rate (SCR) for HI antibody titer at day 21 (ATP cohort for immunogenicity)

Vaccine strain	Group	N	SCR			
			n	%	95% CI	
					LL	UL
A/New Caledonia	FluLD1/1	99	69	69.7	59.6	78.5
	FluLD1/2	99	64	64.6	54.4	74.0
	Fluarix	98	66	67.3	57.1	76.5
A/Wisconsin	FluLD1/1	99	88	88.9	81.0	94.3
	FluLD1/2	99	79	79.8	70.5	87.2
	Fluarix	98	73	74.5	64.7	82.8
B/Malaysia	FluLD1/1	99	76	76.8	67.2	84.7
	FluLD1/2	99	82	82.8	73.9	89.7
	Fluarix	98	65	66.3	56.1	75.6

FluLD1/1 = Low dose influenza vaccine (5 ug HA/strain) with full dose of AS03 adjuvant
FluLD1/2 = Low dose influenza vaccine (5 ug HA/strain) with half dose of AS03 adjuvant
Fluarix = Fluarix vaccine
Seroconversion defined as:
For initially seronegative subjects, antibody titre >= 40 1/DIL after vaccination
For initially seropositive subjects, antibody titre after vaccination >= 4 fold the pre-vaccination antibody titre
N = Number of subjects with pre- and post-vaccination results available
n/% = Number/percentage of seroconverted subjects
95% CI = 95% confidence interval, LL = Lower Limit, UL = Upper Limit

TABLE 8 - Seroconversion factor (SCF) for HI antibody titer at day 21 (ATP cohort for immunogenicity)

Vaccine strain	Group	N	SCF		
			Value	95% CI	
				LL	UL
A/New Caledonia	FluLD1/1	99	14.9	10.4	21.3
	FluLD1/2	99	11.0	7.7	15.9
	Fluarix	98	14.6	9.9	21.6
A/Wisconsin	FluLD1/1	99	16.5	13.0	20.9
	FluLD1/2	99	12.2	9.2	16.1
	Fluarix	98	11.7	8.8	*
B/Malaysia	FluLD1/1	99	13.2	10.0	17.4
	FluLD1/2	99	13.6	10.2	18.0
	Fluarix	98	8.3	6.2	11.0

FluLD1/1 = Low dose influenza vaccine (5 ug HA/strain) with full dose of AS03 adjuvant
FluLD1/2 = Low dose influenza vaccine (5 ug HA/strain) with half dose of AS03 adjuvant
Fluarix = Fluarix vaccine
N = Number of subjects with pre- and post-vaccination results available
SCF = Seroconversion Factor or geometric mean ratio (mean[log10(PI(D21)/PRE)])
95% CI = 95% confidence interval, LL = Lower Limit, UL = Upper Limit

III.7 Safety conclusions

A higher reactogenicity in terms of solicited (local/general) and unsolicited symptoms in the adjuvanted vaccine groups compared to the Fluarix Group was the global trend observed in this study.

A reduction of the AS03 content in the adjuvanted vaccine has a significant impact on all the general and on the local grade 3 symptoms.

The occurrence of unsolicited symptoms tended to be higher in the adjuvanted vaccine groups (55% and 47% of subjects), compared to the Fluarix Group (35%).

From these results, it can be concluded that the reactogenicity and safety profile of the candidate vaccines is satisfactory and clinically acceptable.

III.8. Overall conclusions

III.8.1. Immunogenicity results

The primary objective of this study was to assess humoral immune response (anti-HI antbody titres) elicited by low dose influenza vaccine with two different concentrations of AS03 adjuvant, and by Fluarix.

At Day 21, the three vaccines exceeded the requirements of the European authorities for annual registration of split virion influenza vaccines ("Note for Guidance on Harmonisation of Requirements for influenza Vaccines" for the immuno-logical assessment of the annual strain changes -CPMP/BWP/214/96). GMTs tended to be higher in the adjuvanted groups compared to the Fluarix Group, with a statistically significant difference observed for the A/Wisconsin (FluLD1/1 vs. Fluarix) and B/Malaysia vaccine strains (FluLD1/2 vs. Fluarix). Similar seroprotection rates were observed in all three vaccine groups, ranging from 94.9% to 99%. Seroconversion rates and seroconversion factors were observed to be higher in the adjuvanted groups than in the Fluarix Group. Data from this trial also revealed that the immunogenicity induced by the vaccine with half the dosage of AS03 adjuvant was comparable to that induced with the full dose of adjuvant.

III.8.2. Reactogenicity and safety results

The administration of the low dose influenza candidate vaccine adjuvanted with AS03 was safe and clinically well tolerated in the study population, i.e. adult people aged between 18 and 59 years. The half dose adjuvanted vaccine showed a marked decrease in the incidence of solicited local and general symptoms, compared to the full dose adjuvanted vaccine.

Example IV - Preclinical evaluation of adjuvanted and non-adjuvanted split influenza vaccines (comprising various doses of AS03 adjuvant) in primed BALB/c mice

IV.1. Experimental design and objective

Experiments in influenza-primed mice were performed in order to evaluate the increase in humoral responses by AS03 induced by influenza vaccines formulated with this oil-in-water adjuvant. To simulate the human situation, an experiment was conducted using mice primed with heterosubtypic strains.

IV.1.1. Treatment/group (Table 9)

Groups of 27 adult female BALB/c mice were primed intranasally (20 µl volume) on day 0 with trivalent whole, formalin-inactivated influenza virus (5 µg HA for each strain). Priming strains consisted of earlier drift variants (5 µg HA whole inactivated H1N1 A/Johannesburg/82/96, H3N2 A/Sydney/5/97, B/Harbin/7/94) to those included in the vaccine. Twenty-eight days later, the mice were vaccinated with a single dose of the vaccine candidate intramuscularly in a total volume of 50 µl. Mice were immunized with formulations containing split antigens alone (trivalent split plain) or formulations containing split antigens adjuvanted with two doses of AS03 (full or 1/5). The strains used for the immunizations included H1N1 A/New Caledonia/20/99, H3N2 A/Panama/2007/99, B/Shangdong/7/97 viral antigens (1.5 µg/strain, $1/10^{th}$ of the human dose).

Table 9

Gr	Antigen / Formulation	Other treatment
1	Trivalent split / Plain (non-adjuvanted)	Heterologous priming D0
2	Trivalent split / AS03	Heterologous priming D0
3	Trivalent split / AS03 1/5	Heterologous priming D0

IV.1.2. Preparation of the vaccine formulations

A Premix of Tween 80, Triton X100 and Vitamin E Succinate (VES) is prepared in order to reach a final concentration into the vaccine of 750µg/ml of Tween 80, 110µg/ml of Triton X100 and 100µg/ml of VES. The quantities used in the premix are calculated taking into account the quantities of detergent and VES already present in the strains.

Preparation of one liter of 10 fold concentrated Saline buffer (PBS pH 7.4): to 0.800 l of water for injection, add NaCl 80 g, KCl 2 g, Na_2HPO_4 11.44 g, KH_2PO_4 2 g. After solubilization, adjust to 1.0L with water for injection. pH will be at 7.4 when 10 fold diluted.

Trivalent split / plain

The formulation of one 50µl dose is prepared extemporaneously according the following sequence: Water For Injection + Saline Buffer (10 fold concentrated PBS pH 7.4) + Premix, 5 min magnetic stirring at room temperature, + 1.5µg HA H1N1 strain, 10 min magnetic stirring at room temperature, + 1.5µg HA H3N2 strain, 10 min magnetic stirring at room temperature, + 1.5µg HA B strain, 15 min magnetic stirring at room temperature. The formulations are injected within the hour following the end of their preparation.

Trivalent split /AS03

A Premix of Tween 80, Triton X100 and Vitamin E Succinate (VES) is prepared in order to reach a final concentration into the vaccine of 750µg/ml of Tween 80, 110µg/ml of Triton X100 and 100µg/ml of VES. The quantities used in the premix are calculated taking into account the quantities of detergent and VES already present in the strains.

The formulation of one 50µl dose is prepared extemporaneously according the following sequence: Water For Injection + Saline Buffer (10 fold concentrated PBS pH 7.4) + Premix, 5 min magnetic stirring at room temperature, + 1.5µg HA H1N1 strain, 10 min magnetic stirring at room temperature, + 1.5µg HA H3N2 strain, 10 min magnetic stirring at room temperature, + 1.5µgHA B strain, 15 min magnetic stirring at room temperature, + 25µl SB62 emulsion for the full dose AS03 or 5µl SB62 emulsion for the 1/5 dose AS03, 15 min magnetic stirring at room temperature. The formulations are injected within the hour following the end of their preparation.

IV.1.3. Read-outs (Table 10)

The humoral immune response to vaccination was measured before immunization (day 28) and 14 days after immunization (27 mice/group). Serum samples were tested by the hemagglutination inhibition (HI) test.

Table 10

Read-out	Timepoint	Sample type	Analysis method
Humoral response	D28, D42	Sera	IHA

IV.2. Results

IV.2.1. Humoral immunity

Results are presented in Figure 5. In this mouse model of heterosubtypic priming followed by single vaccination, AS03 and dilutions thereof were shown to induce higher HI titres compared to the plain vaccine. For all influenza A strains, a statistically significant increase of HI titres was observed ($p < 0.05$). For the H1N1 strain, a significant difference in HI titres was also observed between AS03 and AS03 1/5 ($p < 0.05$). A reduced dose of AS03 failed to increase HI titres for all three strains compared to the plain vaccine. Very low responses were observed against the B strain (B/Shangdong); this is likely to be due to the significant antigenic drift between the B strains used for the priming and the vaccine.

IV.3. Summary of results and conclusions

In conclusion, an increase in HI titres was observed in animals primed with hetrosubtypic strains when using AS03 adjuvanted vaccines compared to the plain vaccine. A full dose of AS03 was optimal for obtaining robust HI titres against all three influenza vaccine strains.

Example V - Preclinical evaluation of adjuvanted and non-adjuvanted split influenza vaccines (comprising various doses of AS03 adjuvant) in primed C57Bl/6 mice

V.1. Experimental design and objective

Experiments in influenza-primed mice were performed in order to evaluate the increase in humoral and cellular responses by AS03 induced influenza vaccines formulated with this oil-in-water adjuvant.

To simulate the human situation, an experiment was conducted using mice primed with heterosubtypic strains.

V.1.1. Treatment/group (Table 11)

Groups of 25 adult female C57Bl/6 mice were primed intranasally (20 µl volume) on day 0 with trivalent whole, formalin-inactivated influenza virus (5 µg HA for each strain). Priming

strains consisted of earlier drift variants (5 μg HA whole inactivated H1N1 A/Beijing/262/95, H3N2 A/ Panama/2007/99, B/ Shangdong/7/97) to those included in the vaccine. Twenty-eight days later, the mice were vaccinated with a single dose of the vaccine candidate intramuscularly in a total volume of 100 μl. Mice were immunized with formulations containing split antigens alone (trivalent split plain) or formulations containing split antigens adjuvanted with three doses of AS03 (full, 1/2 or 1/5). The strains used for the immunizations included H1N1 A/New Caledonia/20/99, H3N2 A/New York/55/2004, B/Jiangsu/10/2003 viral antigens (1.5 μg/strain, 1/10th of the human dose).

Table 11

Gr	Antigen / Formulation	Other treatment
1	Trivalent split / Plain (non-adjuvanted)	Heterologous priming D0
2	Trivalent split / AS03	Heterologous priming D0
3	Trivalent split / AS03 1/2	Heterologous priming D0
4	Trivalent split / AS03 1/5	Heterologous priming D0
5	PBS	Heterologous priming D0

V.1.2. Preparation of the vaccine formulations

Trivalent split / plain

The formulations for a 100μl dose are prepared extemporaneously according the following sequence: Water For Injection + Saline Buffer (10 fold concentrated PBS pH 7.4 prepared as taught in exemplelV) + Fluarix Clinical Lot DFLUA014 (1.5μg per strain in the final dose).

Trivalent split /AS03

The formulations for a 100μl dose are prepared extemporaneously according the following sequence: Water For Injection + Saline Buffer (10 fold concentrated PBS pH 7.4 prepared as taught in exemplelV) + Fluarix Clinical Lot DFLUA014 (1.5μg per strain in the final dose)+ 25μl SB62 emulsion for the full dose or 12.5μl SB 62 emulsion for the ½ dose or 5μl SB62 emulsion for the 1/5 dose. The formulations are injected within the hour following the end of the preparation.

V.1.3. Read-outs (Table 12)

The humoral immune response to vaccination was measured 21 days after immunization (10 mice/group) and the serum samples were tested by the haemagglutination inhibition (HI) test. The cellular immune response was tested 7 days post-immunization by intracellular cytokine staining (ICS).

Table 12

Read-out	Timepoint	Sample type	Analysis method
Humoral response	D49	Sera	IHA
Cellular response	D35	PBMCs	ICS

V.2. Results

V.2.1. Humoral immunity (10 mice/group).

Results are presented in Figure 6. In this mouse model of heterosubtypic priming followed by single vaccination, AS03 and dilutions (1/2 and 1/5) thereof were shown to induce higher HI titres compared to the plain vaccine. For all three strains, no difference of HI titres was observed between mice receiving the vaccine adjuvanted with a full dose AS03 or reduced doses AS03.

V.2.2. Cellular immunity (15 mice/group).

Results are presented in Figure 7. Whatever the dilution of AS03, higher CD4+ T cell responses were observed in mice immunized with AS03-adjuvanted trivalent split vaccine compared to mice immunized with trivalent split plain. Compared to the response induced in mice immunized with trivalent split adjuvanted with a full dose AS03, a trend for lower cellular responses was observed when mice were immunized with trivalent split adjuvanted with lower doses of AS03.

V.3. Summary of results and conclusions

In conclusion, an increase in humoral and cellular responses was observed in animals primed with heterosubtypic strains when using AS03 adjuvanted vaccines compared to the plain vaccine. A similar magnitude of humoral response was observed between mice immunized with full dose or fractional doses of AS03 adjuvant. However, a reduction in adjuvant dose was associated with a trend for reduced magnitude of CD4+ T cell response.

Example VI - Preclinical evaluation of the cellular immune response induced by adjuvanted and non-adjuvanted split influenza vaccines (comprising various doses of AS03 adjuvant and low dose antigen) in primed C57Bl/6 mice

VI.1. Experimental design and objective

Experiments in influenza-primed mice were performed in order to evaluate the increase in cellular immune responses by AS03 induced by influenza vaccines containing low dose antigen (0.5 µg/strain, 1/30th human dose) and formulated with this oil-in-water adjuvant. To simulate the human situation, an experiment was conducted using mice primed with heterosubtypic strains.

VI.1.1. Treatment/group (Table 13)

Groups of 15 adult female C57Bl/6 mice were primed intranasally (20 µl volume) on day 0 with trivalent whole, formalin-inactivated influenza virus (5 µg HA for each strain). Priming strains consisted of earlier drift variants (5 µg HA whole inactivated H1N1 A/Beijing/262/95, H3N2 A/ Panama/2007/99, B/ Shangdong/7/97) to those included in the vaccine. Twenty-eight days later, the mice were vaccinated with a single dose of the vaccine candidate intramuscularly in a total volume of 50 µl. Mice were immunized with formulations containing split antigens alone (trivalent split plain) or formulations containing split antigens adjuvanted with three doses of AS03 (full, 1/2 or 1/5). The strains used for the immunizations included H1N1 A/New Caledonia/20/99, H3N2 A/New York/55/2004, B/Jiangsu/10/2003 viral antigens (0.5 µg/strain, 1/30th of the human dose).

Table 13

Gr	Antigen / Formulation	Other treatment
1	Trivalent split / Plain (non-adjuvanted)	Heterologous priming D0
2	Trivalent split / AS03	Heterologous priming D0
3	Trivalent split / AS03 1/2	Heterologous priming D0
4	Trivalent split / AS03 1/5	Heterologous priming D0
5	PBS	Heterologous priming D0

VI.1.2. Preparation of the vaccine formulations

Trivalent split / plain

The formulations for a 50µl dose are prepared extemporaneously according the following sequence: Water For Injection + Saline Buffer (10 fold concentrated PBS pH 7.4 prepared as taught in exemplelV) + Fluarix Clinical Lot DFLUA014 (0.5µg per strain in the final dose).

Trivalent split /AS03

The formulations for a 50µl dose are prepared extemporaneously according the following sequence: Water For Injection + Saline Buffer (10 fold concentrated PBS pH 7.4 prepared as taught in exemplelV) + Fluarix Clinical Lot DFLUA014 (0.5µg per strain in the final dose) + 25µl SB62 emulsion for the full dose or 12.5µl SB 62 emulsion for the ½ dose or 5µl SB62 emulsion for the 1/5 dose. The formulations are injected within the hour following the end of the preparation.

VI.1.3. Read-outs (Table 14)

The cellular immune response was tested 7 days post-immunization by intracellular cytokine staining.

Table 14

Read-out	Timepoint	Sample type	Analysis method
Cellular response	D35	PBMCs	ICS

VI.2. Results

VI.2.1. Cellular immunity

Results are presented in Figure 8. Marginally higher CD4+ T cell responses were observed in mice immunized with trivalent split vaccine adjuvanted with AS03 (full or 1/2 dose) compared to mice immunized with trivalent split plain. Compared to the response induced in mice immunized with trivalent split plain or adjuvanted with a full dose or a half dose of AS03, higher cellular responses were observed when mice were immunized with trivalent split adjuvanted with 1/5 of AS03 dose.

VI.3. Summary of results and conclusions

In conclusion, a minimal increase in CD4+ T cell responses was observed in heterosubtypic primed animals when using AS03 adjuvanted vaccines compared to the plain vaccine. No adjuvant dose response was observed in this experiment and indeed a 1/5 of AS03 dose induced higher frequencies of antigen specific CD4+ T cells than was seen with higher adjuvant doses. Overall these data are not consistent with other preclinical experiments and may be suggestive of a technical issue with this particular experiment.

Example VII - Preclinical evaluation of adjuvanted and non-adjuvanted split H5N1 vaccines (comprising various doses of AS03 adjuvant and antigen) in naïve C57Bl/6 mice

VII.1. Experimental design and objective

Experiments in H5N1-naive mice were performed in order to evaluate the increase in humoral and cellular immune responses by AS03 induced by H5N1 split vaccines formulated with this oil-in-water adjuvant. In the case of a pandemic, it is expected that the entire world population will be immunologically naive to the newly circulating pandemic influenza strain. Due to this naive immune status a pandemic vaccine will likely require two vaccine doses to protect individuals from infection and severe illness caused by a new influenza strain. To represent this lack of previous exposure a naïve mouse model was developed to assess vaccine immunogenicity.

VII.1.1. Treatment/group (Table 15)

Groups of 15 adult female naïve C57Bl/6 mice were immunized on days 0 and 28 with pandemic H5N1 vaccine candidate intramuscularly in a total volume of 50 µl. Mice were immunized with formulations containing split H5N1 antigens alone (H5N1 split plain) or formulations containing split antigens adjuvanted with different doses of AS03 (double, full, 1/2 or 1/5). The strains used for the immunizations included H5N1 A/Vietnam/1194/04 viral antigen (1.5 or 0.38 µg/strain corresponding to$1/10^{th}$ of the human dose).

No formulation was done with a double AS03 dose but rather a concomitant injection of one 50µl H5N1 split/AS03 full dose + one 50µl dose AS03.

Table 15

Gr	Antigen / Formulation	Antigen dose
1	H5N1 split / Plain (non-adjuvanted)	1.5 µg
2	H5N1 split / double dose AS03	1.5 µg
3	H5N1 split / AS03	1.5 µg
4	H5N1 split / AS03 1/2	1.5 µg
5	H5N1 split / AS03 1/5	1.5 µg
6	H5N1 split / Plain (non-adjuvanted)	0.38 µg
7	H5N1 split / double dose AS03	0.38 µg
8	H5N1 split / AS03	0.38 µg
9	H5N1 split / AS03 1/2	0.38 µg
10	H5N1 split / AS03 1/5	0.38 µg
11	PBS	

VII.1.2. Preparation of the vaccine formulations

Preparation of one liter of Final Bulk Buffer (PBS pH 7.2 ± 0.2): to 0.800 l of water for injection, add NaCl 7.699 g, KCl 0.200 g, $MgCl_2$ x $6H_2O$ 0.100 g, Na_2HPO_4 x 12 H_2O 2.600 g, KH_2PO_4 0.373 g. After solubilization, adjust to 1.0L with water for injection

H5N1 split / plain

Preparation of a 50µl dose:

Thiomersal (quantities taking into account its concentration in the strain) and Triton X100 are added to the Final Bulk Buffer. Tween 80 is not added as the content target in the formulation is reach by the Tween concentration of the strain. The final concentrations are of 10µg/ml for Thiomersal, 368 µg/ml for Tween 80 and 35µg/ml for Triton X100 in the 1.5µg formulation dose. They are of 10µg/ml for Thiomersal, 93 µg/ml for Tween80 and 8.9µg/ml for Triton X100 in the 0.38µg formulation dose. After 5-30min magnetic stirring 1.5 or 0.38 µg of HA (H5N1 strain) are added. The formulations are stirred for 30-60 minutes. The pH is checked. Injections occur within the hour following the end of the formulation.

H5N1 split/ AS03

Preparation of a 50µl dose:

Thiomersal (quantities taking into account its concentration in the strain) and Triton X100 are added to the Final Bulk Buffer. Tween 80 is not added as the content target in the formulation is reach by the Tween concentration of the strain. The final concentrations are

of 10µg/ml for Thiomersal, 368 µg/ml for Tween 80 and 35µg/ml for Triton X100 in the 1.5µg formulation dose. They are of 10µg/ml for Thiomersal, 93 µg/ml for Tween80 and 8.9µg/ml for Triton X100 in the 0.38µg formulation dose. After 5-30min magnetic stirring 1.5 or 0.38 µg of HA (H5N1 strain) are added. After 30-60 minutes magnetic stirring, 25 or 12.5 or 5µl of SB62 emulsion is added. The formulations are stirred for 30-60 minutes. The pH is checked. Injections occur within the hour following the end of the formulation

VII.1.3. Read-outs (Table 16)

The humoral immune response was measured 14 days after immunization (10 mice/group) by anti-Ig, IgG1 and IgG2b antibody titers (Figure 9, A-F). The humoral immune response was also measured 21 days after immunization (10 mice/group) by anti-H5N1 hemagglutination inhibition assay (Figure10, A-B).

The cellular immune response was tested 6 days post-immunization (5 pools of 3 mice per group) by intracellular cytokine staining (ICS) of antigen-specific CD4+ T cells numerated by flow cytometry (Figure 11, A-B).

Table 16

Read-out	Timepoint	Sample type	Analysis method
Humoral response	D39	Sera	ELISA, isotypes and HI titers
Cellular response	D34	PBMCs	ICS

VII.2. Results

VII.2.1. Humoral immune response: ELISA and isotypes.

Results are presented in Figure 9.

At each dose of H5N1 split vaccine, all adjuvanted groups induced higher anti-H5N1 Ig, IgG1 and IgG2b antibody titers compared to the non-adjuvanted H5N1 split vaccine (Figures 9 -A to F).

At each dose of H5N1 split vaccine; the anti-H5N1 IgG1 antibody response was 4-5-fold higher than the anti-H5N1 IgG2b antibody response (Figures 9 -C to F). With a dose of 1.5 µg HA of H5N1 split vaccine and combined with each dose of adjuvant, no difference of anti-H5N1 Ig, IgG1 and IgG2b antibody responses were observed (Figures 9-A, C and E).

With a dose of 0.38 µg HA of H5N1 split vaccine, a trend for higher anti-H5N1 Ig titers were obtained after immunization with H5N1 split vaccine adjuvanted with 2x-full dose

compared to the response induced by H5N1 split vaccine adjuvanted with AS03/2 (p=0.7315) and AS03 1/5 (p=0.9744) (Figure 9-B). This trend was also observed for the anti-H5N1 IgG1 antibody response (Figure 9-D). However, the power was not sufficient to observe a statistically significant difference (25% power for 1.7 fold difference, or 47% for a 2 fold difference).

VII.2.2. Humoral immune response: HI titers.

With a dose of 1.5 µg HA/mice:

At each adjuvant dose, all mice immunized with AS03-adjuvanted H5N1 split vaccine induced higher HI titers compared to the response obtained in mice immunized with the non-adjuvanted H5N1 split vaccine (Figure 10-A). No difference of HI titers were observed when H5N1 split vaccine was adjuvanted with a dose range of AS03 (Figure 10-A).

With a dose of 0.38µg HA/dose

At each adjuvant dose, all mice immunized with AS03-adjuvanted H5N1 split vaccine induced higher HI titers compared to the response obtained in mice immunized with the non-adjuvanted H5N1 split vaccine (Figure 10B).

Significantly higher HI titers were observed with H5N1 split vaccine adjuvanted with 2x full dose AS03 compared to the response obtained with H5N1 split vaccine adjuvanted with AS03/2 (p=0.032 for a 4-fold difference) (Figure 10B).

No difference of HI titers was observed in mice immunized with H5N1 split vaccine adjuvanted with 2x full dose AS03 or a full dose AS03 or between mice immunized with H5N1 split vaccine adjuvanted with AS03/2 or AS03/5 (Figure 10B).

Comparison between antigen doses (1.5µg or 0.38µg):

No difference of HI titers were observed between mice immunized with each HA dose of H5N1 split vaccine adjuvanted with AS03, AS03/2 or AS03/5, except between mice immunized with 1.5 µg HA split H5N1 adjuvanted with AS03/5 and mice immunized with 0.38 µg HA split H5N1 adjuvanted with 2x full dose AS03 (Figure 10). HI titers were significantly higher following immunization with 0.38 µg HA split H5N1 adjuvanted with 2x full dose AS03 compared to the higher antigen dose combined with lower adjuvant dose (1.5 µg HA with AS03/5, p=0.0193 for a 4-fold difference) (Figure 10).

VII.2.3. Cellular immune response

Results are presented in Figure 11.

At each dose of H5N1 split vaccine (1.5 or 0.38 µg) higher CD4+ T cell responses were observed in mice immunized with H5N1 split vaccine adjuvanted with various doses of AS03 compared to mice immunized with with the non-adjuvanted H5N1 split vaccine (Figure 11).

At a dose of 1.5 µg H5N1 split vaccine, a reduction of the AS03 doses corresponded to a decrease in CD4+ T cell frequencies (Figure 11A). However, at a dose of 0.38 µg H5N1 split vaccine no difference in CD4+ T cell responses was observed between different adjuvant doses in mice immunized with AS03-adjuvanted H5N1 split vaccines (Figure 11B)..

VII.3. Summary of results and conclusions

Immunogenicity studies in mice showed that adjuvanted H5N1 split vaccine induced significantly higher humoral (anti-H5N1 ELISA and HI titers) and cellular (CD4 + T cells) responses than those induced by the non-adjuvanted H5N1 split vaccine.

No antigen dose response effect was observed for the humoral immune response between mice immunized with 1.5 µg and 0.38 µg adjuvanted H5N1 split vaccine suggesting that in the presence of adjuvant even lower doses of HA may be required to observe a dose response effect in this model.

A strong increase in CD4+ T cell responses was observed in naïve mice when using AS03 adjuvanted H5N1 pandemic vaccines compared to the plain H5N1 vaccine. No impact of the AS03 dilution was observed when a dose of 0.38 µg of H5N1 split vaccine was used as vaccine candidate, while a decrease of CD4 T cell responses was observed when 1.5 µg H5N1 split vaccine was adjuvanted with the reduced dose AS03.

As previously observed, no difference in humoral and cellular immune responses were observed between mice immunized with H5N1 split vaccine (at either antigen dose) adjuvanted with a full dose AS03 or with AS03/2. Some enhancement in the immune response was detected when 2x full dose AS03 was used in the vaccine formulation and accordingly a decrease in the immune response was detected when AS03/5 was used in the vaccine formulation.

Overall, the data reported here support the potency of this novel adjuvant system in this vaccine formulation.

Example VIII - Preclinical evaluation of adjuvanted and non-adjuvanted influenza vaccines in primed Large White pigs

VIII.1. Experimental design and objective

Experiment in influenza-primed pigs was performed in order to evaluate the increase in humoral responses by AS03 induced influenza vaccines formulated with this oil-in-water adjuvant.

Pigs were used in order to evaluate a dose range of AS03 in an animal model close to humans. Pigs show a long list of biological analogies that establish this animal as physiologically the closest to man with very few exceptions (Douglas R., 1972). Moreover, the manifestation of influenza infection in pigs is commonly observed.

VIII.1.1. Treatment/group (Table 17)

Groups of 10 adult Large White female pigs were primed on day 0 with trivalent whole, formalin-inactivated influenza virus (25 μg HA for each strain) intranasally in a total volume of 200 μl. Priming strains consisted of strains homologous to vaccine strains (25 μg HA whole inactivated H1N1 A/New Caledonia/20/99, H3N2 A/Panama/2007/99 and B/Shangdong/7/97). Twenty-eight days later, pigs were vaccinated with a single dose of the vaccine candidate intramuscularly in a total volume of 500 μl. Pigs were immunized with formulations containing split antigens alone (trivalent split plain) or formulations containing split antigens adjuvanted with a dose range of AS03 (full, 1/2 or 1/5). The strains used for the immunizations included H1N1 A/New Caledonia/20/99, H3N2 A/Panama/2007/99 and B/Shangdong/7/97 viral antigens (15 μg HA for H1N1 A/New Caledonia/20/99, H3N2 A/Panama/2007/99 strains and 17.5μg B/Shangdong/7/97 strain as in one human dose).

Groups (10 pigs/group):

Table 17

Gr	Antigen / Formulation	Other treatment
1	Trivalent split / Plain (non-adjuvanted)	Heterologous priming D0
2	Trivalent split / AS03	Heterologous priming D0
3	Trivalent split / AS03 1/2	Heterologous priming D0
4	Trivalent split / AS03 1/5	Heterologous priming D0

VIII.1.2. Preparation of the vaccine formulations

Trivalent split / plain

A Premix of Tween 80, Triton X100 and Vitamin E Succinate (VES) is prepared in order to reach a final concentration into the vaccine of 750µg/ml of Tween 80, 110µg/ml of Triton X100 and 100µg/ml of VES. The quantities used in the premix take into account their content into the strains.

The formulation of one 500µl dose is prepared extemporaneously according the following sequence: Water For Injection + Saline Buffer (10 fold concentrated PBS pH 7.4 prepared as taught in exemple IV) + Premix, 5 min magnetic stirring at room temperature, + 15µg HA H1N1strain, 10 min magnetic stirring at room temperature, + 15µg HA H3N2 strain, 10 min magnetic stirring at room temperature, + 17.5µg HA B strain, 15 min magnetic stirring at room temperature. The formulations are injected within the hour following the end of their preparation.

Trivalent split /AS03

A Premix of Tween 80, Triton X100 and Vitamin E Succinate (VES) is prepared in order to reach a final concentration into the vaccine of 750µg/ml of Tween 80, 110µg/ml of Triton X100 and 100µg/ml of VES. The quantities used in the premix take into account their content into the strains.

The formulation of one 500µl dose is prepared extemporaneously according the following sequence: Water For Injection + Saline Buffer (10 fold concentrated PBS pH 7.4 prepared as taught in exemple IV) + Premix, 5 min magnetic stirring at room temperature, + 15µg HA H1N1 strain, 10 min magnetic stirring at room temperature, + 15µg HA H3N2 strain, 10 min magnetic stirring at room temperature, + 17.5µg HA B strain, 15 min magnetic stirring at room temperature, + 250µl SB62 emulsion for the full dose AS03 or 125µl SB62 emulsion for the 1/2 dose AS03 or 50µl SB62 emulsion for the 1/5 dose AS03, 15 min magnetic stirring at room temperature. The formulations are injected within the hour following the end of their preparation.

VIII.1.3. Read-outs (Table 18)

The humoral immune response to vaccination was measured before intranasal priming (day 0), before immunization (day 28) and 14 days after immunization (10 pigs/group). Serum samples were tested by the haemagglutination inhibition (HI) test.

Table 18

Read-out	Timepoint	Sample type	Analysis method
Humoral response	D0, D28, D42	Sera	IHA

VIII.2. Results and conclusions

VIII.2.1. Humoral immunity

Results are presented in Figure 12. Whatever the dilution of the adjuvant, AS03 adjuvanted trivalent split formulations induced a stronger HI response to all strains than the plain trivalent formulation in this model of homologous priming, although statistical significance was not always reached for all three strains. An adjuvant dose effect was observed with slight differences from strain to strain. For less immunogenic strains such as B/Shangdong, only the trivalent split vaccine adjuvanted with a full dose of AS03 was significantly different from the plain vaccine. In contrast to trivalent split vaccine adjuvanted with a full dose of AS03, a reduced dose of AS03 failed to increase HI titres for all three strains above those seen with the plain vaccine.

Example IX

Immunogenicity of dPly-PhtD-PD antigens adjuvanted with AS03 at different dilutions.

IX.1 Experiment Lims 20080257

dPly (detoxified pneumolysin) and PhtD (polyhistidine triad protein D) are proteins from *Streptococcus pneumoniae* whereas PD is proteins D from *Haemophilus influenzae*. C57bl mice were immunized intramuscularly three times (Day 0, 14 and 28) with 1/10 of a human dose of two lyophilized clinical lots of dPly-PhtD-PD (DSPEA005A and 006A) adjuvanted in AS03A (250 µg SB62) (Groups 1 and 2). A liquid pre-clinical lot of dPly-PhtD-PD in AS03A was used as reference vaccine (Group 3). The clinical lot DSPEA005 reconstituted with AS03B (125 µg SB62) and AS03C (62.5 µg SB62) was also evaluated (Groups 4 and 5). Finally, the two lyophilized clinical lots reconstituted with saline were injected as comparators in order to measure the effect of the adjuvant on the immune response induced against the dPly, PhtD and PD antigens (Groups 6 and 7).

Mice were bled on day 42 and the antibody response directed against each antigen was measured by ELISA. Data are presented in Figure 13 as GMC in µg/ml.

The consistency of the two dPly-PhtD-PD clinical lots was demonstrated (DSPEA005A and 006A). No impact of the lyophilisation was shown (Group 1 and 2 vs Group 3). Significantly higher antibody titers against all antigens were induced by the AS03 formulations (Groups 1 to 5) compared to formulations without adjuvant (Groups 6 and 7). Except for PD in the AS03C formulation (Group 5), no significant difference was observed in the antibody titers induced against each antigen by the formulation containing different concentration of adjuvant (Groups 1, 4 and 5).

IX.2 Experiment Lims 20080334-20080340

C57bl mice were immunized intramuscularly three times (Day 0, 14 and 28) with 1/10 of a human dose of the dPly-PhtD-PD lyophilized clinical lots (DSPEA006A) at different concentrations (15-15-15; 30-30-30 and 60-60-60 µg) and reconstituted in saline or adjuvanted in AS03A (250 µg SB62), AS03B (125 µg SB62) and AS03C (62.5 µg SB62). Mice were bled on day 42 and the antibody response directed against each antigen was measured by ELISA. Data are presented in Figure 14 as GMC in µg/ml.

A significant increase of the antibody titers directed against all antigens was shown in formulation containing AS03 (Groups 1 to 9) compared to formulations containing saline only (Groups 10 to 12). No clear impact of the antigen dose was observed on the immune response directed against each antigen. No significant impact of the adjuvant dilution was observed on the antibody response directed against dPly and PhtD. For PD a significant difference was observed between AS03A and C at the antigen concentration of 3 and 6 µg.

CLAIMS:

1. An immunogenic composition comprising an unconjugated *Streptococcus pneumoniae* protein and an adjuvant composition comprising an oil in water emulsion, wherein said oil in water emulsion comprises 0.5 - 10 mg metabolisable oil, 0.5 – 11 mg tocol and 0.1 – 4 mg emulsifying agent, per human dose.

2. An immunogenic composition comprising an unconjugated *Streptococcus pneumoniae* protein and an adjuvant composition consisting of an oil in water emulsion, wherein said oil in water emulsion comprises 0.5 - 10 mg metabolisable oil, 0.5 – 11 mg tocol and 0.1 – 4 mg emulsifying agent, per human dose.

3. An immunogenic composition comprising an unconjugated *Streptococcus pneumoniae* protein and an adjuvant composition comprising an oil in water emulsion comprising one or more further immunostimulants, wherein said oil in water emulsion comprises 0.5 - 10 mg metabolisable oil, 0.5 – 11 mg tocol and 0.1 – 4 mg emulsifying agent, per human dose.

4. A vaccine composition comprising an unconjugated *Streptococcus pneumoniae* protein and an adjuvant composition comprising an oil in water emulsion, wherein said oil in water emulsion comprises 0.5 - 10 mg metabolisable oil, 0.5 – 11 mg tocol and 0.1 – 4 mg emulsifying agent, per human dose.

5. An immunogenic composition according to claims 1-4 wherein the oil in water emulsion comprises 1-10, 2-10, 3-9, 4-8. 5-7, or 5-6 mg (e.g. 2-3, 5-6, or 9-10mg) metabolisable oil, per human dose.

6. An immunogenic composition according to claims 1-5 wherein the oil in water emulsion comprises 0.5-11, 1-11, 2-10, 3-9, 4-8, 5-7, 5-6 (e.g. 10-11, 5-6, 2.5-3.5 or 1-3 mg) tocol, per human dose.

7. An immunogenic composition according to claims 1-6 wherein the oil in water emulsion comprises 0.1-5, 0.2-5, 0.3-4, 0.4-3 or 2-3 mg (e.g. 0.4-1.2, 2-3 or 4-5 mg) emulsifying agent, per human dose.

8. An immunogenic composition according to claims 1-7 wherein the amount of metabolisable oil is 5.35 mg, per human dose.

9. An immunogenic composition according to claims 1-8 wherein the amount of metabolisable oil is 2.14 mg, per human dose.

10. An immunogenic composition according to claims 1-9 wherein the amount of tocol is 5.94 mg, per human dose.

11. An immunogenic composition according to claims 1-10 wherein the amount of tocol is 2.38 mg, per human dose.

12. An immunogenic composition according to claims 1-11 wherein the amount of emulsifying agent is 2.425 mg, per human dose.

13. An immunogenic composition according to claims 1-12 wherein the amount of emulsifying agent is 0.97 mg, per human dose.

14. An immunogenic composition according to claims 1-13 wherein the metabolisable oil is squalene.

15. An immunogenic composition as claimed in any of claims 1-14 wherein the tocol is alpha-tocopherol.

16. An immunogenic composition as claimed in any of claims 1-15 wherein the emulsifying agent is polyoxyethylene sorbitan monooleate.

17. An immunogenic composition as claimed in claim 16 wherein the polyoxyethylene sorbitan monooleate is selected from the group comprising: Polysorbate® 80 or Tween® 80.

18. An immunogenic composition according to any of the preceding claims wherein the vaccine composition volume is between 0.4 and 1.5 ml

19. An immunogenic composition according to claim 18 wherein said dose volume is 0.5 ml.

20. An immunogenic composition according to claim 18 wherein said dose volume is 0.7 ml.

21. An immunogenic composition according to claim 18 wherein said dose volume is 1.0 ml.

22. The immunogenic composition of any one of claims 1-21 wherein the unconjugated *Streptococcus pneumoniae* protein is selected from the group consisting of of PhtA, PhtD, PhtB, PhtE, pneumolysin, LytB, LytC, LytA, Sp125, SP101, Sp128, Sp130, Sp133 or proteins disclosed in US6699703, including *S. pneumoniae* proteins represented or encoded by SEQ ID NOs: 5225, 5200, 3166, 4360, 5137, 4263, 3166, 5226, 3716, 4360, 5243, 3964, 5179, 3850, 4263, 5137, 5226, 5325, 3850, 5179 or 5325 of US6699703, or immunologically functional equivalent thereof.

23. The immunogenic composition of claim 22 comprising 2 or more unconjugated proteins.

24. The immunogenic composition of claim 23 comprising unconjugated pneumococcal PhtD and unconjugated pneumococcal pneumolysin.

25. A method of treating or preventing pneumococcal disease comprising administering to a patient suffering from or susceptible to pneumoccal infection or disease an immunogenic composition according to any one of claims 1-24.

26. An immunogenic composition according to any one of claims 1 to 24 for use in the prophylactic therapy or therapy of pneumococcal infection or disease.

27. Use of an immunogenic composition according to any of claims 1 to 24 in the manufacture of a medicament for use in prophylactic therapy or therapy of pneumococcal infection or disease.

28. Use of claim 27 wherein the adjuvant composition further comprises one or more immunostimulants.