Vaccine

The present invention relates to novel vaccine formulations, methods of manufacture of such vaccines and the use of such vaccines in the prophylaxis or therapy of disease. In particular the present invention relates to vaccines comprising split enveloped virus preparations.

An enveloped virus is one in which the virus core is surrounded by a lipid-rich outer coat containing viral proteins.

In a particular embodiment the split enveloped virus of the vaccine formulation of the present invention is derived from respiratory syncitial virus (RSV) or parainfluenza virus (PIV). By way of example, RSV is specifically discussed.

Human respiratory syncytial virus is a member of the Paramyxoviridiae family of viruses and causes lower respiratory tract illness, particularly in young children and babies. Recent reports suggest that RSV is also an important pathogen in adults, particularly the elderly.

RSV is an enveloped virus with a non-segmented, negative strand ribonucleic acid (RNA) genome of 15,222 nucleotides that codes for 11 messenger RNAs, each coding for a single polypeptide. Three of the eleven proteins are transmembrane surface proteins: the G (attachment), F (fusion) and SH proteins. One protein is the virion matrix protein (M), three proteins are components of the nucleocapsid (N, P and L), and 2 proteins are nonstructural (NS1 and NS2). There are two further proteins M2-1 and M2-2. Two antigenically distinct sub-groups of RSV exist, designated subgroups A and B. Characterisation of strains from these sub-groups has determined that the major differences reside on the G proteins, while the F proteins are conserved.

Respiratory syncytial virus (RSV) occurs in seasonal outbreaks, peaking during the winter in temperate climates and during the rainy season in warmer climates.

RSV is a major cause of serious lower respiratory tract disease in children. It is estimated that 40-50% of children hospitalised with bronchiolitis and 25% of children hospitalised with pneumonia are hospitalised as a direct result of RSV infections. Primary RSV infection usually occurs in children younger than one year of age; 95% of children have serologic evidence of past infection by two years of age and 100% of the population do so by adulthood.

In infants and young children, infection progresses from the upper to the lower respiratory tract in approximately 40% of cases and the clinical presentation is that of bronchiolitis or pneumonia. Children two to six months of age are at greatest risk of developing serious manifestations of infection with RSV (primarily respiratory failure); however, children of any age with underlying cardiac or pulmonary disease, premature infants, and infants who are immunocompromised, are at risk for serious complications as well.

Symptomatic reinfection occurs throughout life and it has become increasingly apparent that RSV is an important adult pathogen as well, especially for the elderly.

RSV infection is almost certainly underdiagnosed in adults, in part because it is considered to be an infection of children. Consequently, evidence of the virus in adults is not sought in order to explain respiratory illness. In addition, RSV is difficult to identify in nasal secretions from individuals who have some degree of partial immunity to the virus, as do the large majority of adults. Young to middle-age adults typically develop a persistent cold-like syndrome when infected with RSV. Elderly individuals may develop a prolonged respiratory syndrome which is virtually indistinguishable from influenza, with upper respiratory symptoms which may be accompanied by lower respiratory tract involvement, including pneumonia. Institutionalised elderly populations are of particular concern, because they comprise large numbers of susceptible individuals clustered together. The spread of infection through such a population, many of whom have multiple medical problems which may predispose them to a more severe course of the disease, is difficult to control.

Furthermore, reports of recent studies evaluating the impact of RSV infection as a cause of hospitalisation in adults and in community dwelling healthy elderly further point to an important role of RSV infection in severe lower respiratory tract disease in these populations. RSV has been identified as one of the four most common pathogens causing severe lower respiratory tract disease resulting in hospitalisation of adults. It was also demonstrated that serious RSV infections in elderly persons are not limited to nursing homes or outbreak situations. Rather, RSV infection is a predictable cause of serious illness among elderly patients residing in the community. Similar to hospitalisations for influenza A, those related to RSV infections were associated with substantial morbidity, as evidenced by prolonged hospital stays, high intensive care admission rates, and high ventilatory support rates.

These studies point to the medical and economic need for an effective vaccine which can prevent severe complications of RSV infection in infants, adults and the elderly such as community dwelling healthy and institutionalised elderly. Similar considerations apply equally for PIV.

The present invention provides a vaccine formulation comprising a split enveloped virus preparation wherein the virus is respiratory syncitial virus or parainfluenza virus.

Suitably the vaccine formulation further comprises a pharmaceutically acceptable excipient.

The vaccine formulations of the present invention will be derived from enveloped viruses that are capable of being split. The enveloped virus may be derived from a wide variety of sources including viruses from human or animal origin. Suitably the virus is RSVA, RSVB, PIV 1, PIV 2 or PIV3. Where the virus is of animal origin the source is preferably bovine. Where the virus is of animal origin, such as a bovine origin, the virus is preferably a recombinant virus.

The vaccine formulation of the invention optionally comprises another split virus selected from the group consisting of: influenza virus, respiratory syncytial virus,

parainfluenza virus, metapneumovirus, measles virus, mumps virus, Epstein Barr virus, herpes virus, cytomegalovirus, dengue virus, yellow fever virus, tick-borne encephalitis virus, Japanese encephalitis virus, rubella virus, eastern, western and Venezuelan equine encephalitis viruses; and human immunodeficiency virus.

The vaccine formulation of the invention optionally comprises an antigen or antigens from pathogens in combination with the split preparation, to provide additional protection against disease. Suitable antigens, which do not need to come from split preparations, include for example antigens from any of the viruses listed above and pathogens which cause respiratory disease such as *Streptococcus Pneumoniae*.

Preferably the vaccine formulations of the present invention are capable of stimulating a protective immune response against the enveloped virus after delivery.

The splitting of the virus is carried out by disrupting or fragmenting whole virus, infectious (wild-type or attenuated) or non-infectious (for example inactivated), with a disrupting concentration of a splitting agent which is generally, but not necessarily, a surfactant. The virus to be split may also be a chimaeric recombinant virus, having immunogenic elements from more than one different virus. The disruption results in a full or partial solubilisation of all the virus proteins which alters the virus integrity.

Suitably a split virus is obtainable by contacting the PIV or RSV virus with a splitting agent according to the present invention to fully disrupt the viral envelope. Other viral proteins become preferably fully or partially solubilised. The loss of integrity after splitting renders the virus non-infectious which can be assessed by suitable *in vitro* titration assays. Once disrupted the viral envelope proteins are generally no longer associated with whole intact virions. Other viral proteins are preferably fully or partially solubilized and are therefore not associated, or only in part associated, with whole intact virions after splitting.

The effect of the splitting agent on the viral envelope and virus proteins can be followed by the migration of the split virus and viral proteins in sucrose cushion

experiments with visualization by Western Blot analysis and electron microscopy, as described herein.

The preparation of split vaccines according to the invention may involve the further steps of removal of the splitting agents and some or most of the viral lipid material. The process for the preparation of the split enveloped virus may further include a number of different filtration and/or other separation steps such as ultracentrifugation, ultrafiltration, zonal centrifugation and chromotographic steps in a variety of combinations, and optionally an inactivation step e.g. with formaldehyde or β-propiolactone or UV treatment which may be carried out before or after splitting. The splitting process may be carried out as a batch, continuous or semi-continuous process.

The split vaccines according to the invention generally contain membrane fragments and membrane envelope proteins as well as non-membrane proteins such as viral matrix protein and nucleoprotein in the absence of significant whole virions. Split vaccines according to the invention will usually contain most or all of the virus structural proteins although not necessarily in the same proportions as they occur in the whole virus. Preferred split virus preparations comprise at least half of the complement of viral structural proteins, preferably all of such proteins. Subunit vaccines on the other hand consist essentially of one or a few highly purified viral proteins. For example a subunit vaccine could contain purified viral surface proteins which are known to be responsible for eliciting the desired virus neutralising antibodies upon vaccination.

In this invention various splitting agents such as non-ionic and ionic surfactants as well as various other reagents may be used. Examples of splitting agents useful in the context of the invention include:

1. Bile acids and derivatives thereof. Bile acids include cholic acid, deoxycolic acid, chenodeoxy colic acid, lithocholic acid ursodeoxycholic acid, hyodeoxycholic acid and derivatives like glyco-, tauro-, amidopropyl-1-propanesulfonic-, amidopropyl-2-hydroxy-1-propanesulfonic derivatives of forementioned bile acids, or N,N-

bis(3DGluconoamidopropyl) deoxycholamide. A particular example is sodium deoxycholate – NaDOC.

2. Non-ionic surfactants such as octoxynols (the Triton ™ series), polyoxyethylene ethers such as polyoxyethylene sorbitan monooleate (Tween 80 ™), and polyoxythylene ethers or esters of general formula (I):

(I) $HO(CH_2CH_2O)_n$-A-R

wherein n is 1-50, A is a bond or –C(O)-, R is C_{1-50} alkyl or phenyl C_{1-50} alkyl, and combinations of two or more of these. Particular examples are; Tween80™:, Triton X-100™ and laureth 9;

3. Alkylglycosides or alkylthioglycosides, where the alkyl chain is between C6 - C18 typical between C8 and C14, sugar moiety is any pentose or hexose or combinations thereof with different linkages, like 1-> 6, 1->5, 1->4, 1->3, 1-2. The alkyl chain can be saturated unsaturated and/or branched;

4. Derivatives of 3 above, where one or more hydroxylgroups, preferrably the 6 hydroxyl group is/are modified, like esters, ethoxylates, sulfates, ethers, carbonates, sulfosuccinates, Isethionates, ethercarboxylates, quarternary ammonium compounds;

5. Acyl sugars, where the acyl chain is between C6 and C18, typical between C8 and C12, sugar moiety is any pentose or hexose or combinations thereof with different linkages, like 1-> 6, 1->5, 1->4, 1->3, 1-2.The acyl chain can be saturated unsaturated and/or branched;

6. Sulphobetaines of the structure R-N,N-(R1,R2)-3-amino-1-propanesulfonate, where R is any alkyl chain or arylalkyl chain between C6 and C18, typical between C8 and C16. The alkyl chain R can be saturated, unsaturated and/or branched. R1 and R2 alkyl chains between C1 and C4, typically C1;

7. Betains of the structure R-N,N-(R1,R2)-glycine, where R is any alkyl chain between C6 and C18, typical between C8 and C16. The alkyl chain can be saturated

unsaturated and/or branched. R1 and R2 are alkyl chains between C1 and C4, typically C1;

8. Polyoxyethylenealkylether of the structure R-(-O-CH2-CH2-)n-OH, where R is any alkyl chain between C6 and C20 typical between C8 and C14.The alkyl chain can be saturated, unsaturated and/or branched. n is between 5 and 30 typical between 8 and 25;

9. N,N-dialkyl-Glucamides, of the Structure R-(N-R1)-glucamide, where R is any alkyl chain between C6 and C18, typical between C8 and C12. The alkyl chain can be saturated unsaturated and/or branched or cyclic. R1 and R2 are alkyl chains between C1 and C6, typical C1. The sugar moiety might be modified with pentoses or hexoses;

10. Hecameg: (6-0-(N-heptyl-carbamoyl)-methyl-alpha-D-glucopyranoside);

11. Alkylphenoxypolyethoxyethanol of the structure R-C6H4-O-(-CH2-CH2-)n-OH, where R is any alkyl chain between C6 and C18, typical C8. The alkyl chain can be saturated unsaturated and/or branched *(n>=3);*

12. Quaternary ammonium compounds of the structure R, $-N^+$ (-R1, -R2, -R3), where R is any alkyl chain between C6 and C20, typical C20. The alkyl chain can be saturated unsaturated and/or branched. R1, R2 and R3 are alkyl chains between C1 and C4, typical C1;

13. Sarcosyl: N-Laurylsarcosine Na salt;

14. CTAB (cetyl trimethyl ammonium bromide) or Cetavlon.

Most preferred are NaDoc and Sarcosyl. Spliting agents are suitably incubated at room temperature with the virus to be split, for example overnight, to effect splitting. Combinations of splitting agents may be used, as appropriate.

The split vaccine preparation preferably contains at least one surfactant which may be in particular a non-ionic surfactant. The one or more non-ionic surfactants may be residual from the splitting process, and/or added to the virus after splitting. It is believed that the split antigen material is stabilised in the presence of a non-ionic surfactant, though it will be understood that the invention does not depend upon this necessarily being the case. Suitable stabilising non-ionic surfactants include the octoxynols (the Triton ™ series), polyoxyethylene ethers such as polyoxyethylene sorbitan monooleate (Tween 80 ™), and polyoxythylene ethers or esters of general formula (I):

(I) $HO(CH_2CH_2O)_n$-A-R

wherein n is 1-50, A is a bond or –C(O)-, R is C_{1-50} alkyl or phenyl C_{1-50} alkyl, and combinations of two or more of these.

Preferred non-ionic surfactants from the Triton series include Triton X-100 (t-octylphenoxypolyethoxyethanol), Triton X-165, Triton X-205, Triton X-305 or Triton X-405 Triton N-101. Triton X-100 is particularly preferred.

Preferred non-ionic surfactants further include but are not restricted to polyoxyethylene ethers of general formula (I) above in particular: polyoxyethylene-9-lauryl ether, polyoxyethylene-9-stearyl ether, polyoxyethylene-8-stearyl ether, polyoxyethylene-4-lauryl ether, polyoxyethylene-35-lauryl ether, and polyoxyethylene-23-lauryl ether. Most preferably, the polyoxyethylene ether is polyoxyethylene-9-lauryl ether (laureth 9). Alternative terms or names for polyoxyethylene lauryl ether are disclosed in the CAS registry. The CAS registry number of polyoxyethylene-9 lauryl ether is: 9002-92-0. Polyoxyethylene ethers such as polyoxyethylene lauryl ether are described in the Merck index (12th ed: entry 7717, Merck & Co. Inc., Whitehouse Station, N.J., USA; ISBN 0911910-12-3). Laureth 9 is formed by reacting ethylene oxide with dodecyl alcohol, and has an average of nine ethylene oxide units.

Preferably, the final concentration of stabilizing surfactant present in the final vaccine formulation is between 0.001 to 20%, more preferably 0.01 to 10%, and most preferably up to about 2% (w/v). Where one or more surfactants are present, these are

generally present in the final formulation at a concentration of up to about 2% each, generally up to a concentration of about 1% each, typically at a concentration of up to about 0.6% each, and more typically in traces up to about 0.2% or 0.1 % each. Any mixture of surfactants may be present in the vaccine formulations according to the invention.

The enveloped virus may be produced by replication on a suitable cell substrate, in serum or in a serum free process. Tissue culture-grown virus may be produced for example on human cells such as MRC-5, WI-38, HEp-2 or simian cells such as AGMK, Vero, LL_C-Mk_2, LLc-Mk2, FRhL, FRhL-2 or bovine cells such as MDBK, or canine cells such as MDCK, or primary cells such as chicken embryo fibroblasts, or any other cell type suitable for the production of a virus for vaccine purposes including clones derived from the above-mentioned cell lines.

The split vaccine preparation is preferably combined with a pharmaceutically acceptable excipient. The pharmaceutically acceptable excipients used may be those that are conventional in the field of vaccine preparation. The excipients used in any given vaccine formulation will be compatible both with each other and with the essential ingredients of the composition such that there is no interaction which would impair the performance of the ingredients and active agents, if any. All excipients must of course be non-toxic and of sufficient purity to render them suitable for human use. Suitable examples of excipients are well known in the art.

The vaccine formulation may preferably also include an adjuvant which may be a carrier and/or an immunostimulant. The adjuvant may be residual from the splitting process, and/or added to the virus after splitting. Suitable adjuvants for use in the vaccines of the present invention are well known in the art.

Thus a further aspect of the present invention provides a vaccine formulation comprising a split respiratory syncitial virus or split parainfluenza virus vaccine preparation in combination with an adjuvant. Suitably the formulation also comprises a pharmaceutically acceptable excipient.

The form of adjuvant suitable for use generally depends on the means of administration of the vaccine. The vaccine preparations of the present invention may be used to protect or treat a mammal susceptible to, or suffering from disease, by means of administering said vaccine via:

(a) a mucosal route, such as the oral/bucal/intestinal/vaginal/rectal or nasal route;

(b) by parenteral delivery, for example intramuscular, or subcutaneous administration; or

(c) by transdermal, intradermal, intra-epithelial or transcutaneous delivery.

The invention extends to such methods of treatment and protection.

The vaccine preparations of the present invention may optionally be administered by a combination of the routes listed.

Delivery by the mucosal route

Apart from bypassing the requirement for painful injections and the associated negative effect on patient compliance because of "needle fear", mucosal vaccination such as by an intransal method is attractive since it has been shown in animals that mucosal administration of antigens has a good efficiency of inducing protective responses at mucosal surfaces, which is the route of entry of many pathogens. In addition, it has been suggested that mucosal vaccination, such as intranasal vaccination, may induce mucosal immunity not only in the nasal mucosa, but also in distant mucosal sites such as the genital mucosa.

Intranasal administration according to the invention may be in a droplet, spray, or dry powdered form. Nebulised or aerosolised vaccine formulations also form part of this invention. Enteric formulations such as gastro resistant capsules and granules for oral administration, suppositories for rectal or vaginal administration and blisters for bucal or oral administration also form part of this invention.

A preferred mucosal route of administration of the vaccine of the invention is via the intranasal route.

Any suitable adjuvant for intranasal delivery may be used, and in any suitable form, such as a solution, a non-vesicular solution, a suspension or a powder. Preferred adjuvants include those exemplified in WO99/52549 the whole contents of which are incorporated by reference. Preferred adjuvants include, but are not limited to, non-ionic surfactants such as Tween80™, Triton X-100™ and laureth 9, and combinations thereof.

The non-ionic surfactants may advantageously be combined with an immunostimulant such as a non-toxic derivative of lipid A including those described in US 4,912,094 and GB 2,220,211, including non-toxic derivatives of monophosphoryl and diphosphoryl Lipid A such as 3-de-O-acylated monophosphoryl lipid A (3D-MPL) and 3-de-O-acylated diphosphoryl lipid A. A preferred combination is Laureth-9 combined with 3D-MPL. The above immunostimulants may also be used in formulations without non-ionic surfactants, where appropriate.

In a further embodiment of the present invention the adjuvant is an ADP-ribosylating toxin or mutant thereof. Examples of such toxins are the Heat Labile Toxin (LT) from E. coli, and mutants thereof such as LTR192G, and fragments of these toxins such as the ganglioside-binding component (LTB).

Preferred devices for intranasal administration of the vaccines according to the invention are spray devices. Suitable nasal spray devices are commercially available from Becton Dickinson, Pfeiffer GmBH and Valois.

Preferred spray devices for intranasal use do not depend for their performance on the pressure applied by the user. Pressure threshold devices are particularly useful since liquid is released from the nozzle only when a threshold pressure is attained. These devices make it easier to achieve a spray with a regular droplet size. Pressure threshold devices suitable for use with the present invention are known in the art and are described for example in WO 91/13281 and EP 311 863 B. Such devices are currently available from Pfeiffer GmbH and are also described in Bommer, R. Advances in Nasal drug delivery Technology, Pharmaceutical Technology Europe, September 1999, p26-33.

Preferred intranasal devices produce droplets (measured using water as the liquid) in the range 1 to 500μm. Below 10μm there is a risk of inhalation, therefore it is desirable to have no more than about 5% of droplets below 10μm.

Bi-dose delivery is a further preferred feature of an intranasal delivery system for use with the vaccines according to the invention. Bi-dose devices contain two subdoses of a single vaccine dose, one sub-dose for administration to each nostril.

The invention provides in a further aspect a pharmaceutical kit comprising an intranasal administration device as described herein containing a vaccine formulation according to the invention or comprising an intranasal administration device and a separate vaccine formulation for use with that device. The invention also provides an intranasal delivery device comprising a split vaccine formulation of the present invention.

This aspect of the invention is not necessarily limited to spray delivery of liquid formulations. Vaccines according to the invention may be administered in other forms, for example, as a powder.

The vaccines of the present invention may also be administered via the oral route. In such cases the pharmaceutically acceptable excipient may also include alkaline buffers, enteric capsules, microgranules and/or be in the form of blisters.

The vaccines of the present invention may also be administered by the vaginal route. In such cases, the pharmaceutically acceptable excipients may also include emulsifiers, polymers such as CARBOPOL®, and other known stablilisers of vaginal creams and suppositories. The vaccines of the present invention may also be administered by the rectal route. In such cases the excipients may also include waxes and polymers known in the art for forming rectal suppositories.

Parenteral Routes

In addition, the vaccines of the present invention may be parenterally delivered, for example intramuscular, or subcutaneous administration. In this circumstance, an adjuvant which is a preferential stimulator of a TH-1 response is preferred.

An immune response may be broadly distinguished into two categories, being a humoral (antibodies) or a cell mediated immune response (CTLs, T helper cells, NK cells). In both mice and humans, functionally distinct T helper (Th) cell subsets, known as Th1 and Th2 cells are characterized by the patterns of cytokines they produce. Historically, the humoral and cell mediated immune responses have been associated with Th2-type responses and Th1-type responses respectively. These two polarized forms of the specific cellular immune response provide a useful model for explaining the different effector mechanisms involved in the protection against various pathogens. Th1 predominant responses are effective in the eradication of infectious agents, including intracellular pathogens. Th2 responses contribute to the protection against the extracellular forms of the pathogens.

The distinction of Th1 and Th2-type immune responses is not absolute. In reality an individual will support an immune response which is described as being predominantly Th1 or predominantly Th2. The mechanism by which Th cells are responsible for the Th1/Th2 dichotomy was first described in mice when Mosmann and colleagues provided evidence that antigen stimulation of Th cells resulted in the development of restricted and stereotyped patterns of cytokine production (Mosmann, T.R. and Coffman, R.L. (1989) TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annual Review of Immunology, 7, p145-173). In the murine system, Th1 cells produce cytokines such as IFN-γ and promote the activation of antibody-dependent cell cytotoxicity and delayed type hypersensitivity. Th1 cells are also involved in the regulation of the production of IgG2a antibodies. Th2 murine cells produce cytokines such as IL-4 and IL-5 and are involved in the regulation of the humoral responses; more specifically the IgG1 and IgE isotypes.

It is known that certain vaccine adjuvants are particularly suited for the stimulation of either Th1 or Th2-type responses. Traditionally, the best indicators of the Th1:Th2 balance of the immune response after a vaccination or infection include measurement of the production of Th1 or Th2 cytokines by T lymphocytes, and/or the measurement of the antigen specific antibody isotypes, such as the IgG2a:IgG1 ratio in mice.

Thus, a Th1-type adjuvant is one, which stimulates vaccine antigen-specific T-cell populations to produce high levels of Th1-type cytokines. It also induces antigen specific immunoglobulin responses associated with Th1-type isotypes (such as IgG2a) in mice.

Adjuvants which are capable of preferential stimulation of the TH1 cell response are described in International Patent Application No. WO 94/00153 and WO 95/17209.

3 De-O-acylated monophosphoryl lipid A (3D-MPL) is one such adjuvant. This is known from GB 2220211 (Ribi). Chemically it is a mixture of 3 De-O-acylated monophosphoryl lipid A with 4, 5 or 6 acylated chains and is manufactured by Corixa Montana. A preferred form of 3 De-O-acylated monophosphoryl lipid A is disclosed in European Patent 0 689 454 B1 (SmithKline Beecham Biologicals SA).

Preferably, the particles of 3D-MPL are small enough to be sterile filtered through a 0.22micron membrane (as described in European Patent number 0 689 454). 3D-MPL will be present in the range of 10μg - 100μg preferably 25-50μg per dose wherein the antigen will typically be present in a range 2-50μg per dose.

Another preferred adjuvant comprises QS21, an Hplc purified fraction derived from the bark of Quillaja Saponaria Molina. Optionally this may be admixed with 3 De-O-acylated monophosphoryl lipid A (3D-MPL), optionally together with an carrier.

The method of production of QS21 is disclosed in US patent No. 5,057,540.

Non-reactogenic adjuvant formulations containing QS21 have been described previously (WO 96/33739). Such formulations comprising QS21 and cholesterol

have been shown to be successful TH1 stimulating adjuvants when formulated together with an antigen. Thus vaccine compositions which form part of the present invention may include a combination of QS21 and cholesterol.

Further adjuvants which are preferential stimulators of TH1 cell response include immunomodulatory oligonucleotides, for example unmethylated CpG sequences as disclosed in WO 96/02555.

Combinations of different TH1 stimulating adjuvants, such as those mentioned hereinabove, are also contemplated as providing an adjuvant which is a preferential stimulator of TH1 cell response. For example, QS21 can be formulated together with 3D-MPL. The ratio of QS21 : 3D-MPL will typically be in the order of 1 : 10 to 10 : 1; preferably 1:5 to 5 : 1 and often substantially 1 : 1. The preferred range for optimal synergy is 2.5 : 1 to 1 : 1 3D-MPL: QS21.

In one preferrred embodiment of the present invention, the vaccine formulation comprises a vesicular adjuvant formulation comprising cholesterol, a saponin and an LPS derivative. In this regard the preferred adjuvant formulation comprises a unilamellar vesicle comprising cholesterol, having a lipid bilayer preferably comprising dioleoyl phosphatidyl choline, wherein the saponin and the LPS derivative are associated with, or embedded within, the lipid bilayer. More preferably, these adjuvant formulations comprise QS21 as the saponin, and 3D-MPL as the LPS derivative, wherein the ratio of QS21:cholesterol is from 1:1 to 1:100 weight/weight, and most preferably 1:5 weight/weight. Such adjuvant formulations are described in EP 0 822 831 B, the disclosure of which is incorporated herein by reference.

Preferably a carrier is also present in the vaccine composition according to the invention. The carrier may be an oil in water emulsion, lipid structures such as liposomes or micelles or an aluminium salt, such as aluminium phosphate or aluminium hydroxide.

A preferred oil-in-water emulsion comprises a metabolisible oil, such as squalene, alpha tocopherol and Tween 80. Additionally the oil in water emulsion may contain span 85 and/or lecithin and/or tricaprylin.

In a particularly preferred aspect the antigens in the vaccine composition according to the invention are combined with 3D-MPL and alum.

Typically for human administration QS21 and 3D-MPL will be present in a vaccine in the range of 1μg - 200μg, such as 10-100μg, preferably 10μg - 50μg per dose. Typically the oil in water will comprise from 2 to 10% squalene, from 2 to 10% alpha tocopherol and from 0.3 to 3% tween 80. Preferably the ratio of squalene: alpha tocopherol is equal to or less than 1 as this provides a more stable emulsion. Span 85 may also be present at a level of 1%. In some cases it may be advantageous that the vaccines of the present invention will further contain a stabiliser.

Non-toxic oil in water emulsions preferably contain a non-toxic oil, e.g. squalane or squalene, an emulsifier, e.g. Tween 80, in an aqueous carrier. The aqueous carrier may be, for example, phosphate buffered saline.

A particularly potent adjuvant formulation involving QS21, 3D-MPL and tocopherol in an oil in water emulsion is described in WO 95/17210.

Transdermal, intradermal, intra-epithelial or transcutaneous routes

The present invention may also be used to induce an immune response against the viral antigens when applied to the skin (transdermal, intradermal, intra-epithelial or transcutaneous delivery) This includes but is not limited to patches (WO97/48440, WO98/28037, WO99/64580), creams, electroporation-mediated delivery and ballistic delivery such as by compressed gas. The patches may optionally include devices for rupturing skin integrity.

Intradermal delivery may be achieved in humans by, for example, the conventional technique of intradermal injection, the "mantoux procedure". This comprises the 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.

Any suitable adjuvant may be used with the vaccine of the present invention for intradermal delivery. Preferably the adjuvant comprises MPL, QS21 and cholesterol. Preferably the intradermal adjuvant comprises a vesicular adjuvant formulation comprising cholesterol, a saponin and an LPS derivative as described above in respect of parenteral administration. For transdermal delivery the vaccine can also be adjuvanted by the addition of ADP-ribosylating toxins or mutants thereof.

It will be appreciated that any of the above adjuvants which are suitable for any of the vaccination routes described above may also be suitable for use via any other route, and such combinations are specifically and individually included in the present invention.

The formulations of the present invention may be used for both prophylactic and therapeutic purposes. Accordingly, the present invention provides for a method of treating a mammal susceptible to or suffering from an infectious disease, particularly PIV or RSV infection or diseases related to such infection. The method comprises administration to the mammal of an effective amount of a vaccine formulation according to the present invention. In a further aspect of the present invention there is provided a vaccine as herein described for use in medicine.

Vaccine preparation is generally described in New Trends and Developments in Vaccines, edited by Voller et al., University Park Press, Baltimore, Maryland, U.S.A. 1978.

Vaccines may be delivered in any suitable dosing regieme, such as a one dose or two dose regieme. The vaccine may be used in naïve and primed populations.

Where IN delivery is employed, we prefer that the formulation comprises an adjuvant and/or is given to individuals already primed by exposure to RSV or PIV.

The present invention further relates to a method of producing a vaccine formulation which comprises the steps of

(a) splitting an enveloped virus;

(b) optionally admixing the split enveloped virus preparation with a stabilising agent; and

(c) optionally admixing the split enveloped virus preparation with an adjuvant (carrier and/or immunostimulant).

Preferably the virus is RSV or PIV. Suitably the method comprises steps (a) and (b), (a) and (c), or (a) (b) and (c). Suitably the stabilising agent comprising at least one surfactant selected from the group comprising polyoxyethylene sorbitan monooleate (TWEEN80™); t-octylphenoxypolyethoxyethanol (TRITON X100™); polyoxyethylene-9-lauryl ether.

Optionally the vaccine produced in this way is admixed with a carrier.

The present invention is illustrated by, but not limited to, the following Examples and Figures, wherein:

Fig 1 illustrates a Western Blot of split RSVA with an anti F antibody;
Fig 2 illustrates a Western Blot of split RSVA with an anti-M2 antibody;
Fig 3 illustrates a Western Blot of split RSVA with an anti G antibody;
Fig 4 illustrates a Western Blot of split RSVA with an anti N antibody;
Fig 5 illustrates RSV/A starting material visualised by EM;
Fig 6 illustrates RSV/A split with NaDOC visualised by EM;
Fig 7 illustrates RSV/A split with Sarkosyl visualised by EM;
Fig 8 illustrates Anti-FG Antibody (ELISA) Titers (Post II) in Primed Mice Immunized with Split RSV by the Intramuscular or Intranasal Routes;

Fig 9 illustrates Anti-RSV/A Neutralizing Antibody Titers (Post II) in Primed Mice Immunized with Split RSV by the Intramuscular or Intranasal Routes;

Fig 10 illustrates Anti-FG IgG Isotype Responses (Post II) in Primed Mice Immunized with Split RSV by the Intramuscular or Intranasal Routes;

Fig 11 illustrates Anti-FG Antibody (ELISA) Titers (Post I) in Primed Mice Immunized with Split RSV by the Intramuscular or Intranasal Routes;

Fig 12 illustrates Anti-FG Antibody (ELISA) Titers in Unprimed Mice Immunized with Split RSV by the Intramuscular Route;

Fig 13 illustrates anti-RSV/A neutralising antibody titres in unprimed mice immunized with split RSV by the intramuscular route;

Figure 14 illustrates .Anti-FG IgG Isotype Responses in Unprimed Mice Immunized with Split RSV by the Intramuscular Route;

Figure 15 illustrates Anti-FG Antibody (ELISA) Titers in Unprimed Mice Immunized with Split RSV by the Intranasal Route;

Fig 16 illustrates Anti-RSV/A Neutralizing Antibody Titers in Unprimed Mice Immunized with Split RSV by the Intranasal Route;

Fig 17 illustrates Anti-FG Antibody (ELISA) Titers in Primed Guinea Pigs; Immunized with Split RSV by the Intradermal or Intramuscular Routes; and

Fig 18 illustrates Anti-RSV/A Neutralizing Antibody Titers in Primed Guinea Pigs Immunized with Split RSV by the Intradermal Route.

Example 1 Generation of Split Viruses

Enveloped viruses derived from a variety of virus families are split by addition of splitting agents such as surfactants. The splitting is evaluated by characterization of the migration of the split viruses in sucrose gradients or cushions with visualization by SDS-PAGE/Western blot analysis and by direct examination of split viral products using electron microscopic evaluation.

The split viruses described in this example include representatives of a variety of enveloped viral families. For example, members of the Paramyxoviridae family (respiratory syncytial viruses A and B, parainfluenza virus-3, mumps, and measles

virus), Togaviridae family (rubella virus), and the Herpesviridae family (Epstein Barr virus, cytomegalovirus, or herpes simplex virus) are evaluated.

The act of disrupting the viral particle (splitting) is accomplished by addition of a splitting agent such as a surfactant at solubilizing concentrations to the cell-free viral preparations. In particular, bile acids and alkylglycosides are used as surfactants. The surfactants, alone or in various combinations, are added and incubated to allow the process to go to completion. All viruses are stored pending evaluation by electron microscopy.

Evaluation of efficient splitting is conducted initially using sucrose gradient or cushion centrifugation. Briefly, surfactant-treated and non-treated samples are applied to sucrose gradients/cushions and the fractions analysed on SDS-PAGE gels. Migration of all types of virion proteins in the soluble fractions indicate efficient splitting. Samples deemed efficiently split by the sucrose-SDS-PAGE analysis are further analyzed by electron microscopy. The samples are visualized using standard negative staining techniques.

The following specific splitting experiments were carried out on RSV and PIV

1.1 Cell culture conditions

Human wild-type RSV/A/Long and PIV-3 were replicated in VERO cells in a stationary serum free process. Before infection, VERO cells were grown for 4 days to confluency. Virus production conditions were adapted to each virus : MOI 0.03, 4 days for RSV/A and MOI 0.01, 5 days for PIV-3 at 37°C. At the day of harvest, cell fluids were recovered after lysis and addition of stabiliser and were immediately stored at –70°C.

1.2 Virus purification

After clarification by centrifugation at 1,000 x g for 10 min, virus particles were pelleted from the supernatant by a PEG 6000 precipitation. The pellet was

resuspended in Tris 50 mM-NaCl 50 mM-MgSO4 2 mM pH 7.5 buffer followed by a benzonase treatment. This solution was ultrafiltrated on a 500 kD AGT membrane against 5 volumes of phosphate-buffered saline then diafiltred against 5 volumes of phosphate buffer pH 7.5.

Intact viral particles were produced as confirmed by EM and centrifugation on a sucrose cushion as described herein. The protein concentration was determined.

1.3 Virus splitting

The viral particles were split by addition of a splitting agent to the cell-free viral preparation.

To be effective a detergent must be used above its critical micellar concentration, cmc. All detergents were used at a final concentration above their cmc value. The ratio D/P (detergent/ protein ratio) was studied. The splitting was achieved successfully with a ratio D/P ≥25, which is preferred.

The following detergents were used at a 2% concentration to split the virus particles; Sodium Deoxycholate, Sarkosyl, Plantacare and Laureth 9.

After splitting, the solutions were dialyzed against formulation buffer (PO4 10 mM/ NaCl 150 mM pH7.5) for removal of excess detergent.

The splitting process is summarised below:

RSV-A : Virus purification flow sheet.

Harvest Clarification

Centrifugation in Beckman JA10 rotor at 3500 RPM (1000 x g) + 4°C for 10 min.

→ **Clarified supernatant.**

↓

10 % PEG 6000 precipitation.

Slow stirring 1h30 at +4°C
Centrifugation 3500 RPM (1000 x g) – 20 min
Pellet resuspended in Tris 50mM – NaCl 50 mM- MgSO4 2mM pH 7.5 buffer.

↓

Benzonase treatment.
At 125 unit/ml
Minimum 4 hours incubation under stirring

↓

ULTRAFILTRATION : AGT – VAGE4A – 500 Kd - 420 cm²

5 vol against PO4(Na) 10 mM – NaCl 150 mM pH 7.5
followed by 5 volumes of PO4 (Na) 20 mM pH 7.5

↓

Splitting

Detergent addition => ≤2% final – incubation O/N at room temperature under slow stirring

↓

Clarification

Dead-end filtration on Sartopure 300 (GF2 – depth filter 1.2 μ m)

↓

ULTRAFILTRATION : detergent elimination - concentration

5 vol PO4(Na) 20 mM pH 7.5
then 5 vol PO4(Na)10 mM / NaCl 150 mM pH 7.5

↓

Split bulk

1.4 Split virus characterisation

Integrity of starting viruses and split quality was determined by ultracentrifugation on a 30% sucrose cushion (1h at 50.000 rpm in TL100 Beckman rotor). Fractions were analyzed by specific Western blotting assays; electron microscopy and infectivity titer were performed on some of these fractions.

1.4.1 Ultracentrifugation:

After half filling a centrifuge tube with the 30% sucrose solution (450 µl), the sample (450 µl) to be analyzed was laid gently and carefully onto this sucrose cushion then run for 1 hour at 50.000 rpm at +4°C in a Beckman TL100 rotor. After centrifugation, the tube was drained in 3 parts The upper phase (300 µl) is referred to as the 'supernatant'. The middle phase (300 µl) is the interface phase between the sample and the sucrose cushion, called herein the 'middle'. The lower phase (300µl) is the bottom solution with the resuspended pellet when centrifugation has been performed on integer virus; called the 'pellet'.

These 3 fractions were further analysed.

1.4.2 Western blotting analysis:

This analysis allows the integrity of the virus to be checked (pellet fraction positive) and the efficacy of the split to be determined (suitably, supernatant fraction positive for all or most strcutural proteins such as the envelope proteins).

Specific antibodies were used for the characterization of specific viral proteins.

For RSV-A non-split and split fractions were analyzed for the anti F protein (surface protein); anti G protein (surface protein); anti N protein (nucleocapsid) and anti M protein (matrix) content.

For PIV-3 virus, the non-split and split fractions were analyzed for their HN protein content with a monoclonal antibody and the F, M, HN proteins content with a polyclonal antibody.

1.4.3 Criteria for splitting

The presence of a positive Western Blot (WB) signal against all four proteins tested in the pellet fraction and absence of a signal in the two other fractions before splitting suggests the presence of whole intact virus in the viral preparation.

The split was considered effective when the envelope was disrupted, and envelope proteins were detected in the supernatant and/or middle fraction. For RSV, splitting was effective when F or G, for example, were detected in S or M fractions. Preferably F and G were located substantially in the S and/or M layers, and not in the pellet.

1.5 Summary of results:

Results are shown in Figures 1-4 for RSVA.

In all western blot results, 'Split –O' means the virus before splitting. 'S', 'M' and 'P' refer to 'Supernatant', 'Middle' and 'Pellet' samples taken after ultracentrifugation on the sucrose cushion respectively. Numbering of lanes is left to right. Volumes refer to quantity of sample deposited on the SDS-PAGE gels.

Fig 1 illustrates a western blot of split RSVA probed with mAb B4 (anti-F).

In the upper panel:

1	STD	10 µl
2	Split - O	10 µl
3	Split O - S	10 µl
4	Split O - M	10 µl
5	Split O - P	10 µl
6	Split DOC - S	10 µl
7	Split DOC - M	10 µl
8	Split DOC - P	10 µl
9	Split sarco - S	10 µl
10	Split sarco - M	10 µl
11	Split sarco - P	10 µl

In the lower panel:

1	STD	10 µl
2	Sample buffer	10 µl
3	Split O - S	10 µl
4	Split O - M	10 µl
5	Split O - P	10 µl
6	Split planta - S	10 µ
7	Split planta - M	10 µl
8	Split planta - P	10 µl
9	Split laureth9 - S	10 µl
10	Split laureth9 - M	10 µl
11	Split laureth9 - P	10 µl
12	STD	10 µl

Fig 2 illustrates a western blot of split RSVA probed with an anti-M monoclonal;

In the upper panel:

1	STD	10 µl
2	Split - O	20 µl
3	Split O - S	20 µl
4	Split O - M	20 µl
5	Split O - P	20 µl
6	Split DOC - S	20 µl
7	Split DOC - M	20 µl
8	Split DOC - P	20 µl
9	Split sarco - S	20 µl
10	Split sarco - M	20 µl
11	Split sarco - P	20 µl

Lower panel:

1	STD	10 µl
2	Sample buffer	20 µl
3	Split O –S	20 µl
4	Split O – M	20 µl
5	Split O – P	20 µl
6	Split planta – S	20 µl
7	Split planta – M	20 µl
8	Split planta – P	20 µl
9	Split laureth9 – S	20 µl
10	Split laureth9 – M	20 µl
11	Split laureth9 - P	20 µl

Fig 3 illustrates a western blot of split RSVA probed with an anti-G monoclonal;

Upper panel:

1	STD	10 µl
2	Split - O	20 µl
3	Split O - S	20 µl
4	Split O - M	20 µl
5	Split O - P	20 µl
6	Split DOC - S	20 µl
7	Split DOC - M	20 µl
8	Split DOC - P	20 µl
9	Split sarco - S	20 µl
10	Split sarco - M	20 µl
11	Split sarco - P	20 µl

Lower panel:

1	STD	10 µl
2	Sample buffer	20 µl
3	Split O –S	20 µl
4	Split O – M	20 µl
5	Split O – P	20 µl
6	Split planta - S	20 µl
7	Split planta – M	20 µl
8	Split planta – P	20 µl
9	Split laureth9 – S	20 µl
10	Split laureth9 – M	20 µl
11	Split laureth9 - P	20 µl

Fig 4 illustrates a western blot of split RSVA probed with an anti-N monoclonal;

Upper panel:

1	STD	10 µl
2	Split - O	20 µl
3	Split O - S	20 µl
4	Split O - M	20 µl
5	Split O - P	20 µl
6	Split DOC - S	20 µl
7	Split DOC - M	20 µl
8	Split DOC - P	20 µl
9	Split sarco - S	20 µl
10	Split sarco - M	20 µl
11	Split sarco - P	20 µl

Lower panel:

1	STD	10 µl
2	Sample buffer	20 µl
3	Split O – S	20 µl
4	Split O – M	20 µl
5	Split O – P	20 µl
6	Split planta – S	20 µl
7	Split planta – M	20 µl
8	Split planta – P	20 µl
9	Split laureth9 – S	20 µl
10	Split laureth9 – M	20 µl
11	Split laureth9 - P	20 µl

The presence of a signal in the medium and supernatant fractions and hardly any band in the pellet fraction after splitting for the F and G proteins shows that the viral envelope was completely disrupted. The presence of a signal in all fractions for the N and M proteins shows the presence of these proteins in the split preparations. These results suggest that all four detergents tested lead to RSVA split virus.

Analysis of the signals against all four proteins in all fractions and in particular the comparative signals against N and M proteins after splitting in the medium and supernatant fractions suggests that, in the conditions tested, NaDOC and Sarkosyl not only lead to split virus but are also able to disrupt all viral structures and solubilize structural and non-structural proteins.

Similar results were obtained with split PIV.

NaDoc and Sarkosyl are preferred splitting agents for all viruses.

1.6 In vitro viral titrations

The loss of integrity after splitting renders the virus non-infectious. Analysis of the successful disruption of RSVA and PIV3 is shown by the loss of 10^6 log or more in viral titer following splitting.

1.7 Electron microscopy analysis

Electron microscopy analysis was performed using a standard two-step negative staining method using Na phosphotungstate as contrasting agent (Hayat and Miller, 1990, Negative Staining, McGraw, ed. Hill). Grids were examined to assess the splitting pattern of the material.

Analysis by electron microscopy of non-split and NaDoc or Sarkosyl split RSVA and PIV3 virus preparations supports the observations made by Western Blot analysis. To illustrate results, the RSV data are shown. Figures 5 illustrates RSVA virus prior to

splitting. Figures 6 and 7 show RSVA virus split with NaDOC or Sarkosyl respectively.

The non-split virus (whole intact virus) contained relatively well preserved or lightly damaged viral particles and some amorphous material. NaDoc or Sarkosyl split viruses showed the appearance of a heterogeneous spread of amorphous material, aggregated to various extent, with few identifiable structures from viral envelope or nucleoproteic origin. Similar data were obtained with PIV.

Example 2. Immunogenicity of split vaccines in mice.

Split RSV and/or PIV preparations are used as immunogens to vaccinate mice to assess the immunogenicity of these preparations. Briefly, 8 week old female mice are immunized with the split vaccine preparations. Parenteral , mucosal and ID routes of immunization are investigated. Adjuvants vary depending on the route of administration. In all cases a non-adjuvanted control is included. Two doses are given at an interval of several weeks.

Two weeks following the final dose, the animals are sacrificed and blood, spleen cells, and/or nasal washes are collected. The virus-specific humoral immune response in serum is assessed by testing the mouse serum in virus-specific ELISA assays. In addition, the isotype profile of the antibody response is determined using Isotype-specific assays. The presence of neutralizing antibodies in the serum is assessed using a specific virus neutralization assay. Induction of a relevant local immune response may be assessed by assay of neutralizing antibodies in the nasal washes or alternatively assay of virus-specific IgA in the nasal washes.

Induction of virus specific cellular immune responses is assessed by in vitro stimulation of harvested spleen cells and measurement of cellular proliferation (tritiated thymidine uptake) and/or secretion of IL-5 and IFNγ by the stimulated cells.

The impact of the variables in the experiment can be assessed with specific attention paid to the quality and magnitude of the response induced by the split formulations.

Split RSV was tested by way of example.

2.1 Split RSV formulations

The following series of experiments exemplifies that split RSV induces a potent immune response when administered by the intramuscular (IM), intranasal (IN), or intradermal (ID) routes. In order to more accurately reflect the immune status of either a pediatric (naive) or elderly (primed) population, the immunogenicity was evaluated in either primed or unprimed animals and immunogenicity was demonstrated in both populations.

In the first set of experiments, 8 week old female Balb/c mice were used to test the immunogenicity of the split RSV preparation administered by either the IM or IN routes. Priming was accomplished by administration of 3×10^5 plaque forming units (pfu) of live RSV virus administered intranasally in a volume of 60 µl (2 X 30 µl). Three weeks following priming, animals were vaccinated with RSV split antigen. Quantitation of the RSV split product was based on an RSV F protein specific ELISA which quantitates the F protein in the split product compared to a recombinant FG protein standard. Group A mice were immunized with 2 doses of RSV split antigen containing 4.2 µg F protein in 100 µl administered by the intramuscular route at a 21 day interval. Group B mice were immunized with 2 doses of RSV split antigen containing 4.2 µg F protein adjuvanted with 50 µg $Al(OH)_3$ administered in 100 µl by the intramuscular route at a 21 day interval. Group C mice were immunized with a first dose of RSV split antigen containing 2.7 µg F protein in 60 µl and a second dose administered 21 days later of RSV split antigen containing 4 µg F protein in 60 µl by the intranasal route. Two weeks following the last dose all animals were sacrificed and the immune response evaluated.

The results of the experiment are summarized in Figures 8-18. The first immune read outs used to evaluate the immune response were ELISA assays which measure the total RSV FG-specific immunoglobulin (Ig) or the FG-specific IgG isotypes (IgG_1 and IgG_{2A}) present in the sera of vaccinated animals. In these assays 96 well dishes are coated with recombinant RSV FG antigen and the animal sera are serially diluted and applied to the coated wells. Bound antibody is detected by

addition of a biotinylated anti-mouse Ig, IgG_1, or IgG_{2A}, followed by an amplification with peroxidase-conjugated streptavidin. Bound antibody is revealed upon addition of OPDA substrate, followed by treatment with 2 N H_2SO_4 and measurement of the optical density (OD)at 490 nm. The antibody titer is calculated from a reference using SoftMax Pro software (using a four parameter equation) and expressed in EU/ml.

In addition to ELISA assays, neutralization assays were included to further characterize the quality of the immune response induced by the immunizations. For the neutralization assay, two-fold dilutions of animal sera were incubated with RSV/A virus (3000 pfu) and guinea pig complement for 1 hour at 37°C in 96 well tissue culture dishes. Hep-2 cells (10^4 cells/well) were added directly to each well and the plates incubated for 4 days at 37°C. The supernatants were aspirated and a commercially available WST-1 solution was added to each well. The plates were incubated for an additional 18-24 hours at 37°C. The OD was monitored at 450 nm and the titration analysed by linear regression analysis. The reported titer is the inverse of the serum dilution which resulted in 50% reduction of the maximal OD observed for uninfected cells.

Figure 8 shows the results obtained using the total Ig ELISA read out. In primed mice a potent anti-FG antibody response was induced by 2 vaccinations with split RSV antigen administered IM (Groups A,B) or IN (Group C). For IM vaccination there was a trend towards heightening the magnitude of the immune response upon adjuvantation with $Al(OH)_3$.

Specifically, Figure 8 shows anti-FG antibody (ELISA) titers (post secondary vaccination) in mice primed with live RSV and immunized with split RSV by the intramuscular (IM) or intranasal (IN) routes. Group A received 2 doses of 4.2 μg each split RSV IM. Group B received 2 doses of 4.2 μg each split RSV adjuvanted with alum IM. Group C received 2 doses of 2.7 and 4.0 μg respectively split RSV IN.

Figure 9 shows the results of the neutralization assay for the Fig 8 samples. A potent virus neutralizing antibody response was induced in these primed animals by either

IM or IN vaccination with 2 doses of the split RSV product and a similar trend for the boosting of the response in the alum adjuvanted group as noted with the ELISA read out.

Specifically, Figure 9 shows Anti-RSV/A Neutralizing antibody titers (post secondary vaccination) in mice primed with live RSV and immunized with split RSV by the intramuscular (IM) or intranasal (IN) routes. Group A received 2 doses of 4.2 μg each split RSV IM. Group B received 2 doses of 4.2 μg each split RSV adjuvanted with alum IM. Group C received 2 doses of 2.7 and 4.0 μg respectively split RSV IN.

Figure 10 shows the results of the isotype analysis for the Fig. 8 and 9 samples. In animals primed intranasally the ratio of IgG_{2a}:IgG_1 is increased compare to subsequent data (Figure 14) generated in unprimed mice, suggesting a tendency towards a more Th1-like response when mice are primed with live virus (i.e. natural situation in elderly populations).

Specifically, Figure 10 shows Anti-FG IgG Isotype (ELISA) responses (post secondary vaccination) in mice primed with live RSV and immunized with split RSV by the intramuscular (IM) or intranasal (IN) routes. Group A received 2 doses of 4.2 μg each split RSV IM. Group B received 2 doses of 4.2 μg each split RSV adjuvanted with alum IM. Group C received 2 doses of 2.7 and 4.0 μg respectively split RSV IN.

Figure 11 demonstrates that even after a single dose of antigen that a strong immune response in generated in response to IM or IN vaccination with split RSV in primed populations. Thus, in primed populations split RSV is a potent immunogen inducing high titer antibody responses following either IM or IN vaccination.

Specifically, Figure 11 shows Anti-FG antibody (ELISA) titers (post primary vaccination) in mice primed with live RSV and immunized with split RSV by the intramuscular (IM) or intranasal (IN) routes. Group A received 2 doses of 4.2 μg each split RSV IM. Group B received 2 doses of 4.2 μg each split RSV adjuvanted with alum IM. Group C received 2 doses of 2.7 and 4.0 μg respectively split RSV IN.

Group D was primed only and did not receive a vaccination – antibody titers reported for this group are 21 days post-priming.

In the second series of experiments unprimed mice were used to document the effect of antigen dose and adjuvantation on the immunogenicity of the split RSV product. Mice were immunized with split RSV antigen containing 4.2 (high dose) or 0.42 μg (low dose) F protein administered in 100 μl by the IM route. The IM split RSV formulations at both antigen doses were either adminstered without adjuvant or adjuvanted by addition of 50 μg $Al(OH)_3$, or a vesicular adjuvant formulation comprising cholesterol, 3dMPL and QS21 as described in EP0822831, herein referred to as DQ 3DMPL. A control group received whole purified RSV virus containing 3.4 μg F protein also administered in 100 μl by the IM route. A second dose of each formulation (all identical to first injection except the whole purified virus group which received a preparation containing 4.2 μg of F protein) was administered IM 30 days later.. Two weeks following the last dose all animals were sacrificed and the immune response evaluated.

Figure 12 summarizes the FG-specific Ig response in these animals. A potent immune response was induced in animals immunized with either high or low dose F protein when adjuvanted with DQ/3DMPL and administered by the IM route. Lower levels of antibody were induced by the alum adjuvanted or unadjuvanted formulations administered by the IM route. In all cases there was an apparent dose response as the low antigen dose induces lower levels of antibody than the high antigen dose. The levels of antibody induced by the high dose of whole virus were similar to that induced by low dose of RSV split adjuvanted with DQ/3DMPL.

Specifically Figure 12 shows Anti-FG antibody (ELISA) titers (post secondary vaccination) in unprimed mice immunized with split RSV by the intramuscular (IM) route. Group A received 2 doses of 0.42 μg each split RSV. Group B received 2 doses of 0.42 μg each split RSV adjuvanted with alum. Group C received 2 doses of 0.42 μg each split RSV adjuvanted with DQ/3DMPL. Group D received 2 doses of 4.2 μg each split RSV. Group E received 2 doses of 4.2 μg each split RSV adjuvanted with alum. Group F received 2 doses of 4.2 μg each split RSV adjuvanted

with DQ/3DMPL. Group G received 2 doses of 3.4 and 4.2 μg respectively whole purified RSV virus.

Similar results were obtained when the virus neutralization read out was used on the samples of Fig 12 (Figure 13). Figure 13 shows Anti-RSV/A Neutralizing antibody titers (post secondary vaccination) in unprimed mice immunized with split RSV by the intramuscular (IM) route. Group A received 2 doses of 0.42 μg each split RSV. Group B received 2 doses of 0.42 μg each split RSV adjuvanted with alum. Group C received 2 doses of 0.42 μg each split RSV adjuvanted with DQ/3DMPL. Group D received 2 doses of 4.2 μg each split RSV. Group E received 2 doses of 4.2 μg each split RSV adjuvanted with alum. Group F received 2 doses of 4.2 μg each split RSV adjuvanted with DQ/3DMPL. Group G received 2 doses of 3.4 and 4.2 μg respectively whole purified RSV virus.

Analysis of the IgG isotypes induced by these formulations (Figure 14) revealed that while unadjuvanted and alum adjuvanted formulations induce a typically Th2-like response in unprimed mice, addition of DQ/3DMPL to the formulation results in shift towards a more Th1-like response with an increased IgG_{2a}:IgG_1 ratio. This is similar to the profile observed in primed animals vaccinated with the unadjuvanted or alum adjuvanted formulations (Figure 10). Thus, in unprimed mice IM vaccination with split RSV induces strong antibody responses which can be augmented by addition of adjuvants.

Specifically, Figure 14 shows anti-FG IgG Isotype (ELISA) responses (post secondary vaccination) in unprimed mice immunized with split RSV by the intramuscular (IM) route. Group A received 2 doses of 0.42 μg each split RSV. Group B received 2 doses of 0.42 μg each split RSV adjuvanted with alum. Group C received 2 doses of 0.42 μg each split RSV adjuvanted with DQ/3DMPL. Group D received 2 doses of 4.2 μg each split RSV. Group E received 2 doses of 4.2 μg each split RSV adjuvanted with alum. Group F received 2 doses of 4.2 μg each split RSV adjuvanted with DQ/3DMPL. Group G received 2 doses of 3.4 and 4.2 μg respectively whole purified RSV virus.

Similar immunizations were done in unprimed mice by the IN route. In this case mice received split RSV antigen containing 2.4 μg F protein (delivered in 60 μl – 2 X 30 μl) for the first dose. For the second dose delivered 30 days later the mice received split RSV antigen containing 3.5 μg F protein. The IN split RSV were either adminstered without adjuvant or adjuvanted by addition of 5 μg *E. coli* labile toxin (LT) or with polyoxyethylene-9-lauryl ether 0.5% (herein 'Laureth 9'). A control group was immunized intranasally with whole purified RSV virus containing 2.0 μg F protein in the first dose and 3.5 μg F protein in the second dose. Two weeks after the final vaccination the animals were sacrificed and the immune response evaluated.

As shown in Figures 15 and 16 antibody responses are induced by the IN formulations in unprimed mice. While the ELISA read out (Figure 15) suggests that the responses to IN vaccination are slightly weaker than those induced by IM, the neutralization read out (Figure 16) suggests that the LT adjuvanted split RSV IN formulation is at least as good as IM in inducing neutralizing antibodies and that the other formulations are also comparable to IM. Thus, in unprimed mice split RSV administered by the IN route is also immunogenic,

Specifically, Figure 15 shows anti-FG antibody (ELISA) titers (post secondary vaccination) in unprimed mice immunized with split RSV by the intranasal (IN) or intramuscular (IM) routes. Group A received 2 doses of 2.4 and 3.5 μg each split RSV IN. Group B received 2 doses of 2.4 and 3.5 μg each split RSV adjuvanted with Laureth 9 IN. Group C received 2 doses of 2.4 and 3.5 μg each split RSV adjuvanted with LT IN. Group D received 2 doses of 2.0 and 3.5 μg each purified whole virus IN. Group E received 2 doses of 4.2 μg each split RSV IM.

Figure 16 shows anti-RSV/A Neutralizing antibody titers (post secondary vaccination) in unprimed mice immunized with split RSV by the intranasal (IN) or intramuscular (IM) routes. Group A received 2 doses of 2.4 and 3.5 μg each split RSV IN. Group B received 2 doses of 2.4 and 3.5 μg each split RSV adjuvanted with Laureth 9 IN. Group C received 2 doses of 2.4 and 3.5 μg each split RSV adjuvanted

with LT IN. Group D received 2 doses of 2.0 and 3.5 μg each purified whole virus IN. Group E received 2 doses of 4.2 μg each split RSV IM.

The immunogenicity of the RSV split antigen when administered by ID route was evaluated in guinea pigs. The feasibility of true ID injection in this species has been confirmed by injection of India ink into the dermis and histological examination of the tissues (data not shown). Again, in an effort to simulate the immune status found in elderly populations (i.e. primed against RSV), the Hartley guinea pigs (5 per group) were primed either with live RSV virus (5×10^5 pfu administered IN in 100 μl – 50 μl/nostril; Groups A-E) or with purified whole RSV virus (containing 6 μg F protein administered IM in 100 μl; Groups F-J). Two equivalent doses of vaccine were administered at Day 21 and Day 42 post priming. Groups A and F received the split RSV preparation containing 4.2 μg of F protein administered by the ID route. Groups B and G received the split RSV preparation containing 0.84 μg of F protein administered by the ID route. Groups C and H received the split RSV preparation containing 4.2 μg of F protein adjuvanted with DQ/3DMPL administered by the ID route. Groups D and I received the split RSV preparation containing 0.84 μg of F protein adjuvanted with DQ/3DMPL administered by the ID route. Groups E and J received the split RSV preparation containing 4.2 μg of F protein administered by the IM route. Animals were bled 3 weeks after the first dose of vaccine and 2 weeks after the second dose of vaccine and the immune response evaluated.

The results of this experiment are summarized in Figures 17 and 18. Figure 17 shows the FG specific immune response detected in the guinea pig sera during the course of the experiment. The FG-specific ELISA used for guinea pig testing was similar to the mouse ELISA in that wells were coated with recombinant FG and the animal sera diluted and applied to coated wells. However, in this assay bound antibody is detected by horse radish peroxidase conjugated anti-guinea pig Ig, followed by addition of substrate and revelation as previously described. For all groups a weak response to priming is observed, allowing a clear observation of a boosted response on vaccination by either the ID or IM route. A boost is clearly observed upon primary vaccination and an additional boost is evident following secondary vaccination in all groups except Group H where the titers have plateaued. A clear increase in the

magnitude of the response is observed upon adjuvantation (Groups C,D, H, I). No clear effect of antigen dose is observed in this experiment and 1/5 dose administered by the ID route seems to function as well as a full dose administered by the IM route even without adjuvantation. Figure 18 summarizing the neutralization data shows similar trends. Thus, in a primed population (primed either by live virus infection or administration of purified whole virus) a single dose of split RSV administered by the ID route is strongly immunogenic and this response can be further boosed by a second dose of vaccine.

Specifically, Figure 17 shows Anti-FG antibody (ELISA) titers in primed Guinea Pigs immunized with split RSV by the intradermal (ID) or intramuscular (IM) Routes. Groups A-E were primed with live RSV virus. Groups F-J were primed with purified whole virus. For vaccination Groups A and F received 2 doses 0.84 μg each split RSV ID. Groups B and G received 2 doses of 4.2 μg each split RSV ID. Groups C and H received 2 doses 0.84 μg each split RSV adjuvanted with DQ/3DMPL ID. Groups D and I received 2 doses 4.2 μg each split RSV adjuvanted with DQ/3DMPL ID. Groups E and J received 2 doses of 4.2 μg each split RSV IM.

Figure 18 shows Anti-RSV/A Neutralizing antibody titers in primed Guinea Pigs immunized with split RSV by the intradermal (ID) or intramuscular (IM) Routes. Groups A-E were primed with live RSV virus. Groups F-J were primed with purified whole virus. For vaccination Groups A and F received 2 doses 0.84 μg each split RSV ID. Groups B and G received 2 doses of 4.2 μg each split RSV ID. Groups C and H received 2 doses 0.84 μg each split RSV adjuvanted with DQ/3DMPL ID. Groups D and I received 2 doses 4.2 μg each split RSV adjuvanted with DQ/3DMPL ID. Groups E and J received 2 doses of 4.2 μg each split RSV IM.

In summary, these experiments have demonstrated that split RSV antigen is strongly immunogenic in both naive and primed populations. In addition, these experiments have shown that split RSV can be administered by a variety of routes including intramuscular, intranasal, and intradermal and in all cases is immunogenic. Addition of adjuvants can boost the antibody responses in some cases and/or induce a shift in the profile of the response from a Th2-like to a Th1-like response.

CLAIMS:

1. A vaccine formulation comprising a split enveloped RSV virus preparation, wherein the split enveloped virus preparation comprises viral membrane fragments, viral membrane envelope proteins, viral matrix and nucleoproteins.

2 A vaccine formulation as claimed in claim 1 wherein the vaccine preparation additionally comprises another split virus selected from the group consisting of: influenza virus, respiratory syncytial virus, parainfluenza virus, metapneumovirus, measles virus, mumps virus, Epstein Barr virus, herpes virus, cytomegalovirus, dengue virus, yellow fever virus, tick-borne encephalitis virus, Japanese encephalitis virus, rubella virus, eastern, western and Venezuelen equine encephalitis viruses; and human immunodeficiency virus.

3 A vaccine formulation as claimed in claim 1 or 2 which additionally comprises one or more residual splitting agents.

4 A vaccine formulation as claimed in Claim 3 wherein the residual splitting agent is selected from the group consisting of: laureth 9, NaDOC, Sarcosyl group, Tween 80™ and Triton X100™.

5 A vaccine formulation according to Claim 4 wherein the residual splitting agent is NaDOC or Sarkosyl.

6 A vaccine formulation as claimed in any one of Claims 1 - 5 which additionally comprises a stabilising agent

7 A vaccine formulation as claimed in Claim 6 wherein the stabilising agent is a surfactant.

8 A vaccine formulation as claimed in claim 7 wherein the surfactant is either singly or a mixture of polyoxyethylene sorbitan monooleate (TWEEN80™), t-octylphenoxypolyethoxyethanol (TRITON X100™) and polyoxyethylene-9-lauryl ether.

9 A vaccine formulation as claimed in any one of the preceding claims wherein the vaccine is formulated to be delivered intranasally.

10 A vaccine formulation as claimed in any one of Claims 1 – 8 wherein the vaccine is formulated to be delivered intramuscularly or subcutaneously.

11 A vaccine formulation as claimed in any one of claims 1-8 wherein the vaccine is formulated to be delivered via the transdermal, intradermal, intra-epithelial or transcutaneous route.

12 A vaccine formulation according to claim 11, wherein the vaccine is formulated for intradermal delivery.

13 A vaccine formulation as claimed in any one of the preceding claims which additionally comprises an adjuvant.

14 A vaccine formulation according to claim 13 wherein the adjuvant is polyoxyethylene-9-lauryl ether.

15 A vaccine formulation according to claim 13 wherein the adjuvant is a preferential stimulator of TH1 cell response.

16 A vaccine formulation as claimed in claim 15 wherein the preferential stimulator of TH1-cell response is selected from the group of adjuvants comprising: 3D-MPL, QS21, a mixture of QS21 and cholesterol, a CpG oligonucleotide and combinations thereof.

17 A vaccine formulation according to claim 16 wherein the adjuvant is a vesicular adjuvant formulation comprising cholesterol, a saponin and an LPS derivative.

18 A vaccine formulation as claimed in any preceding claim which additionally comprises a carrier.

19 A method of producing a vaccine formulation as claimed in any of claims 1 to 18 which comprises the steps of

(a) splitting an RSV enveloped virus;

(b) optionally admixing the split enveloped virus preparation with a stabilising agent; and

(c) optionally admixing the split enveloped virus preparation with an adjuvant.

20 A method of producing a vaccine formulation as claimed in Claim 19 wherein the split virus preparation is admixed with a stabilising agent, the stabilising agent comprising at least one surfactant selected from the group comprising polyoxyethylene sorbitan monooleate (TWEEN80™); t-octylphenoxypolyethoxyethanol (TRITON X100™); polyoxyethylene-9-lauryl ether.

21 Use of a split RSV vaccine preparation in the manufacture of a vaccine as claimed in any of claims 1 to 18.

22 Use of a split RSV vaccine preparation in the manufacture of a vaccine formulation as claimed in any of claims 1 to 18 for the prophylaxis or treatment of disease.

23 A kit for delivery of an intranasal vaccine formulation as claimed in any one of claims 1 - 18 comprising:
(a) a split RSV enveloped virus preparation; and
(b) an intranasal delivery device.

24 An intranasal delivery device comprising a split vaccine formulation according to any of claims 1-18.

25 A method for protecting or treating a mammal susceptible to, or suffering from disease caused by RSV, the method comprising administering an effective amount of a vaccine according to any of claims 1-18.

26 A method according to claim 25, wherein the vaccine is administered by intradermal or intranasal route.

27 Use of a split RSV vaccine preparation in the manufacture of a vaccine formulation as claimed in any of claims 1 to 18 for intranasal or intradermal delivery.

28 A formulation, method, use, kit, or device according to any preceding claim, wherein the vaccine formulation is immunogenic.

29 A formulation, method, use, kit, or device according to claim 28, wherein the vaccine formulation is immunogenic in both seropositive and seronegative individuals.