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SUPERINDUSTRIA Y COMERCIO
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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

Santa Fe de Bogotá, D.C.

02-04-09

Re.: Expediente No. 00 - 28.931

Solicitud de Patente

SMITHKLINE BEECHAM BIOLOGICALS s.a

Debidamente traducidas adjunto acompaño las solicitudes de patente básica Inglesa No. 9909077.1 y No. 9915106.0 la cual le solicito agregar a este expediente para los efectos legales respectivos.

De Usted atentamente,

GERMAN CASTILLO GRAU

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Novel Compositions

This invention relates to novel vaccine formulations, methods for preparing them and their use in therapy. In particular the present invention relates to combination vaccines for administration to children and the elderly.

Streptococcus pneumoniae is a gram positive bacteria responsible for considerable morbidity and mortality, particularly in the young and aged. Expansive colonisation of the respiratory tract, and middle ear, especially in young children, is the single most common cause for hospital visits in the US. The bacteria may become invasive, infecting the lower lungs and causing pneumonia. The rate of pneumococcal pneumonia in the US for persons over 60 years of age is estimated to be 3 to 8 per 100,000. In 20% of cases this leads to bacteremia, and other manifestations such as meningitis, with a mortality rate close to 30% even with antibiotic treatment. There are 90 known serotypes of *Streptococcus pneumoniae* which are determined by the structures of the capsular polysaccharide surrounding the bacteria, and this is its major virulence factor.

A 17 - valent pneumococcal vaccine (Moniarix) is known, based on the purified polysaccharides of the pneumococcal serotypes most commonly involved in invasive disease. The method of purification of these polysaccharides was disclosed in European Patent 72513 B1. Vaccine efficacy trials with lower valent vaccines demonstrated a 70 to 90% efficacy with respect to serotypes present in the combination. Case controlled studies in the US in persons >55 years using a 14 valent vaccine demonstrated 70% efficacy (Mills, O.F., and Rhoads, G.G; J. Clin. Epidemiol. (1996); Vol.49(6) 631-636). Inclusion of additional polysaccharides (to make a 23-valent pneumococcal vaccine) were accepted on the basis of an adequate serological response, even though there was clinical efficacy data lacking (K. R. Brown In 'Combined.Vaccines and Simultaneous Administration', Ed. Williams et al. New York Academy of Sciences 1995 pp 241-249).

Simultaneous immunisation with a 23-valent pneumococcal vaccine (Pnu-Immune 23 - Lederle USA) and influenza vaccine (Fluarix) has been studied (TJ Fletcher, TW Tunnicliffe, K Hammond, K Roberts, JG Ayres; (1997) Br. Med. J. 314: 1663) and no significant immunological differences were noted between simultaneous immunisation, or immunisations one month apart.

Pneumococcal polysaccharides can be rendered more immunogenic in infants by chemically coupling them to protein carriers, and clinical efficacy trials are being performed to verify this concept for efficacy in preventing infant Otitis media.

Infants primed with a 7 to 11-valent conjugate pneumococcal vaccine may be boosted with the 23-valent plain polysaccharide pneumococcal vaccine in order to increase the serotype coverage and IgG concentrations (Block, SL, JA. Hedrick, R.A. Smith, R.D. Tyler, M. Giordani, M.D. Blum, J. Sadoff, E. Keegan; Abstract G-88, 37th ICAAC, Toronto (1997); Anderson, E.L., Kennedy, D.J., Geldmacher, K.M., Donnelly, J., Mendelman, P; The Journal of Paediatrics, May 1996. Vo. 128, n°5, Part 1, 649-653).

Respiratory Syncytial virus (RSV) occurs in seasonal outbreaks, peaking during the winter in temperate climates and during the rainy season in warmer climates (DeSilva LM & Hanlon MG. Respiratory Syncytial Virus: a report of a 5-year study at a children's hospital. *J Med Virol* 1986;**19**:299-305). Wherever the area, RSV tends to have a regular and predictable pattern and other respiratory viral pathogens that occur in outbreaks are rarely present concurrently (Glezen WP & Denny FW. Epidemiology of acute lower respiratory disease in children. *N. Engl. J. Med.* 1973;**288**:498-505).

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

70 74

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 (Dowell SF, Anderson LJ, & Gary Jr HE, *et al. J. Infect Dis* 1996;**174**:456-462; Falsey AR, Cunningham CK, & Barker WH, *et al. J. Infect Dis* 1995;**172**:389-394). Dowell identified RSV as one of the four most common pathogens causing severe lower respiratory tract disease resulting in hospitalisation of adults (Dowell SF, Anderson LJ, & Gary Jr HE, *et al. J. Infect Dis* 1996;**174**:456-462). Falsey 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 (Falsey AR, Cunningham CK, & Barker WH, *et al. J. Infect Dis* 1995;**172**:389-394).

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 both community dwelling healthy and institutionalised elderly.

SmithKline Beecham has undertaken the development of an RSV vaccine based on the extracellular domain of the F and G surface glycoproteins of RSV strain A. The use of an adjuvant is of primary importance for a subunit recombinant vaccine. Alum is the only adjuvant currently licensed for human use. However, Alum has a limited adjuvant effect

on the humoral response, and is known to induce mainly a TH2 cellular response (Byars NE, Nakano G, & Welch M, *et al. Vaccine* 1991;9:309-318). It has been found, however, that 3 De-O-acylated monophosphoryl lipid A (3D-MPL) in combination with Alum improves the humoral response and stimulates preferentially a TH1-type immunity. The Aluminium salt with 3D-MPL adjuvant is denoted Alum/3D-MPL.

The present invention provides a vaccine composition comprising:

(a) one or more *Streptococcus pneumoniae* polysaccharides either conjugated to a protein or peptide, or non-conjugated; and
(b) an RSV antigen
in combination with an adjuvant which is a preferential stimulator of TH1 cell response.

The trend towards combination vaccines has the advantage of reducing discomfort to the recipient, facilitating scheduling, and ensuring completion of regiment; but there is also the concomitant risk of reducing the vaccine's efficacy. It would be, therefore, advantageous to make vaccine combinations which meet the needs of a population, and which, in addition, do not interfere with each other. It would be of further advantage if the combination of the vaccines results in synergy with resulting improvement of one or both vaccines efficacy, or improved correlates of protections for one or both vaccines. This is achieved by the vaccine composition of the invention which is of great benefit for administration to children or the elderly who may be particularly at risk of *Streptococcus pneumoniae*, and/or RSV infection.

Optionally the vaccine composition of the invention additionally comprises one or more of a number of other antigens such as an antigen against *influenza* virus. Currently available influenza vaccines include whole inactivated virus vaccines, split particle vaccines, and subunit vaccines. Inactivated influenza vaccines, of all kinds, are usually trivalent vaccines. They contain antigens derived from two influenza A virus strains and one

71 72

influenza B strain. A standard 0.5ml dose of most of them contain 15μg of haemagglutinin component from each strain.

The procedure for determining the strains to be incorporated into an influenza vaccine is a complex process which involves collaboration between the World Health Organisation, national health authorities and vaccine manufacturers.

There are two conjugation methods generally used for producing immunogenic polysaccharide constructs: (1) direct conjugation of carbohydrate and protein; and (2) indirect conjugation of carbohydrates and protein via a bifunctional linker or spacer reagent. Generally, both direct and indirect conjugation require chemical activation of the carbohydrate moiety prior to derivatisation. See for example US 5,651,971 and Dick & Beurret, "Glycoconjugates of Bacterial Carbohydrate Antigens," Conjugate Vaccines, J.M. Cruse & R.E. Lewis (eds), Vol. 10, 48 - 114 (1989).

The *Streptococcus pneumoniae* polysaccharides, if conjugated, are conjugated to either a protein or a peptide. Polysaccharide antigens have been conjugated to a number of T helper proteins. These provide T-helper epitopes. Representative proteins include Diphtheria Toxoid, Tetanus toxoid, and protein D or its lipidated derivative lipoprotein D from Heamophilius influenzae B. Other suitable protein carriers include, Diphtheria Crm 197 and the major non structural protein from influenzae, NS1 (particularly amino acid 1-81).

The *Streptococcus pneumoniae* polysaccharide in the composition of the invention is preferably a vaccine with a number of valencies, preferably at least an 11-valent vaccine, for example a 17- or 23-valent vaccine. Most preferably the 23-valent pneumococcal polysaccharide or the 11-valent conjugate vaccine.

Suitable RSV antigens for inclusion in vaccines include an inactivated RSV virus, such as a formalin inactivated RSV virus, or antigens derived from the RSV virus, preferably human RSV envelope glycoproteins, such as the RSV F or G protein or immunogenic fragments

thereof as disclosed for example in US Patent 5149650, or a chimeric polypeptide comprising at least one immunogenic fragment from both RSV F and G proteins, advantageously an RSV FG chimeric protein as disclosed for example in US patent 5194 595 preferably expressed from CHO cells.

The RSV antigen in the composition of the invention is preferably an RSV FG chimeric protein.

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 Ribi Immunochem Montana. A preferred form of 3 De-O-acylated monophosphoryl lipid A is disclosed in International Patent Application No. 92/116556.

Preferably, the size of the particles of 3D-MPL are small enough to be sterile filtered through a 0.22micron membrane (as described in European Patent number 0689454).

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 non-toxic 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.

QS21 is a Hplc purified non-toxic fraction of a saponin from the bark of the South American tree Quillaja Saponaria Molina and its method of its production is disclosed (as QS21) 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.

Preferably a carrier is also present in the vaccine composition according to the invention. The carrier may be an oil in water emulsion, or alum.

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 preferred aspect aluminium hydroxide (alum) or aluminium phosphate will be added to the composition of the invention to enhance immunogenicity.

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 1 μg - 200 μ g, such as 10-100 μ g, preferably 10 μ g - 50 μ g 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 or less than 1 as this provides amore 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.

The vaccine of the present invention will contain an immunoprotective quantity of the antigens and may be prepared by conventional techniques.

Vaccine preparation is generally described in Pharmaceutical Biotechnology, Vol.61 Vaccine Design - the subunit and adjuvant approach, edited by Powell and Newman, Plenum Press, 1995; New Trends and Developments in Vaccines, edited by Voller et al., University Park Press, Baltimore, Maryland, U.S.A. 1978. Encapsulation within liposomes is described, for example, by Fullerton, U.S. Patent 4,235,877. Conjugation of proteins to macromolecules is disclosed, for example, by Likhite, U.S. Patent 4,372,945 and by Armor et al., U.S. Patent 4,474,757.

The amount of protein 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. Generally, it is expected that each dose will comprise 1-1000 mg of protein, preferably 2-100 mg, most preferably 4-40 mg. An optimal amount for a particular vaccine can be

ascertained by standard studies involving observation of antibody titres and other responses in subjects. Following an initial vaccination, subjects may receive a boost in about 4 weeks.

In addition to vaccination of persons susceptible to *Streptococcus pneumoniae* or RSV infections, the pharmaceutical compositions of the present invention may be used to treat, immunotherapeutically, patients suffering from the said infections.

In a further aspect of the present invention there is provided a method of manufacture as herein described, wherein the method comprises mixing a *Streptococcous pneumoniae* antigen and an RSV antigen with a Th-1 inducing adjuvant, for example 3D-MPL and, preferably, a carrier, for example alum.

If desired, other antigens may be added, in any convenient order, to provide multivalent vaccine compositions as described herein.

In the examples that follow, evaluation of the effect of combining the RSV vaccine candidate, FG with the commercially available 23-valent pneumococcal vaccine (Pneumune from Wyeth-Lederle) in a mouse model was carried out. This model tested a combined immunisation where the vaccines are physically mixed and injected in the same site. The results of this study demonstrate a mutual synergy, resulting in improved responses for certain immunological markers for both vaccines.

EXAMPLES

1. Formulation process:

H_2O-diluted FG antigen (2µg:1/10 HD) was adsorbed for 15 min on 50 µg of $Al(OH)_3$. When used, 5µg of 3D-MPL were added to the preparation as a suspension of 100 nm particles and incubated for 30 min. Formulations were then buffered with 10-fold concentrated PBS pH 7.4. When used, the 23 valent pneumococcal vaccine (11.5µg:1/50 HD) was added 15 min. after the addition of the buffer solution. For groups without FG the same formulation sequence was followed so that the 3D-MPL was first adsorbed on $Al(OH)_3$ before adding the 23 valent pneumococcal vaccine. Fifteen minutes after the addition of the concentrated buffer or the 23 valent pneumococcal vaccine phenoxyethanol (5mg/ml) was added to the formulations as preservative.

All incubations were carried out at room temperature with agitation. The formulations were prepared simultaneously for the 2 injections with a 7-day maturation of the finalised formulations before the first injection.

Composition of formulation constituents:

COMPONENT	BATCH NUMBER	CONCENTRATION (µg/ml)	BUFFER
FG	54/023	265	10 PO4, 150NaCl pH6.8
Al $(OH)_3$	96A0089	10380	H_2O
23-Valent	Pneumune	1150	saline
3D-MPL	109	964	H_2O

2. Immunization protocol:

11 groups of 10 mice were immunised by different routes (50 µl) at days 0 and 28 with various formulations (see ***Table 1***). Group 7 and 8 were immunised with live RSV by the intra-nasal route (60µl). Sera were obtained at days 28 (28 d Post I) and 42 (14 d Post II). On day 42, spleen and node cells were taken from 5 mice of groups 4, 5, 6, 7, 8, 9, as well as from 5 naïve Balb/c mice (group 1, not immunised).

74 75

3. Humoral response.

3.1. Anti-FG antibodies:

All humoral results were performed for 10 mice/group (individual response for the anti-FG titers and pooled sera for the isotype profile) and cellular results were presented for 5 mice/group.

Individual sera were obtained 28 days after the first Immunization and 14 days after the second immunisation and were tested for the presence of FG specific Ig antibodies and their isotype (IgG2a, IgG1) distribution.
The assay protocol was as follows: coating overnight at 4°C with 50 µl of purified FG 54/023 (1µg/ml) per well, saturation 1h at 37°C, incubation with sera 1h30 at 37°C, incubation with anti-mouse Ig biotin 1/1500 (or IgG1, IgG2a biotin 1/1000) 1h30 at 37°C, incubation with strepta-peroxydase 1/2500 30 min at 37°C, incubation with OPDA Sigma 15 min at RT, stop with H_2SO_4 2N.
OD were monitored at 490 nm and the titers determined by linear regression (y=a.logx + b): titer = serum dilution giving 50% reduction of the maximal OD. 95% confidence limits (IC 95%) were calculated for each group.

Individual sera obtained 14d Post II were tested for the presence of neutralising antibodies using the following protocol: 50 µl of serial two-fold dilutions of sera (first dilution 1/250) were incubated for 1 hour at 37°C with 50 µl of a mixture containing 500 pfu of RSV-A/Long (Lot 14) and guinea pig complement in a 96 well plate in duplicate. 100 µl of a HEp-2 cell suspension at 10^5 cells/ml were then added to each well and the plates were incubated for 4 days at 37°C in the presence of 5% CO_2.
The supernatants were then aspirated, and after addition of a 100 µl of a WST-1 preparation (dilution 1/12.5) the plates were further incubated for 24H at 37°C in the presence of 5% CO_2. The OD were monitored at 595 nm and the titers determined by linear regression (y=a.logx + b): titer = serum dilution giving 50% reduction of the maximal OD observed for the uninfected cells.
Controls in test included a pool of randomly chosen human sera (Human pool) and Sandoglobuline (lot 069, generic human IgG produced by Sandoz).

3.2. Anti-Pneumococcal Polysaccharide IgG:

Murine IgG to pneumococcal polysaccharides types 6B, 14, 19F and 23F was measured by ELISA in a method adapted from the CDC protocol. This protocol includes the addition of soluble cell wall polysaccharide (CPS) to the sera to inhibit the measurement of CPS antibodies. CPS is a phosphoryl choline containing teichoic acid common to all pneumococci. It is present under the capsule, and antibodies to it are only weakly

protective. Since CPS is linked to the capsular polysaccharide, there is usually 0.5 to 1% CPS contaminating the purified capsular polysaccharide used to coat the ELISA plates. Thus, measurement of the CPS antibodies can confound the interpretation ELISA results with respect to the capsular polysaccharide.
The ELISA was performed with polysaccharides coated at 20, 5, 10 and 20 μg/ml in carbonate buffer for types 6B, 14, 19F and 23F respectively. Sera was pre-mixed with the equivalent of 500 μg/ml CPS in undiluted sera, and incubated for 30 minutes before addition to the ELISA plate. Murine IgG was detected with Jackson ImmunoLab goat anti-murine IgG (H+L) peroxidase at 1:2000 dilution. The titration curves were referenced to polysaccharide specific murine monoclonal antibodies of known concentration for each serotype using logistic log comparison by SoftMax Pro. The monoclonals used were HASP4, PS14/4, PS19/5 and PS23/22 for types 6B, 14, 19F and 23F respectively. Due to the limited quantity of sera available, pooled sera was tested, thus statistical analysis is not available.

4. Cellular response

Spleen and lymph node cells were isolated 14d Post II from groups 4-9 and from naïve mice (Group 1) for use as a negative control for the FG-specific cellular response analysis. Samples were analysed for both FG-specific lymphoproliferation and cytokine (IFN-γ + IL-5) secretion.
Proliferation was evaluated after a 96h incubation of $4x10^5$ cells/well of 96 well plates with 200 μl of media containing 10 to 0.03 μg/ml of FG (3-fold dilutions). Upon ^{3}H-thymidine incorporation, the FG specific proliferation was measured following our standard protocol. Cytokine induction was evaluated after 96 h incubation of $2.5x10^6$ cells per well of 24 well with 1 ml of media containing 10μg to 0.01μg of FG (10-fold dilutions). Supernatants were then harvested to determine the quantity of IFN-γ and IL-5 induced by ELISA following our standard protocol.

Results

1. GROUPS.

10 groups received two immunisations of various formulations containing either the 23-valent vaccine or FG or a combination of both formulated with Aluminium hydroxide and 3D-MPL or Aluminium hydroxide. Group 1 constitutes the control for the CMI studies. Groups 2 and 9 will allow the evaluation of the immunogenicity of the 23 Valent pneumoccocal vaccine upon intraperitoneal immunization which was used in the literature for the evaluation of 23 Valent pneumoccocal vaccine, and upon IM immunization, Groups 3 and 10 allow a parallel analysis to groups 2 and 9 except that the 23 Valent pneumoccocal vaccine is formulated with Alum/3D-MPL. Groups 2 and 3 also constitute

controls for the impact of Alum/3D-MPL on the 23 Valent pneumoccocal vaccine when it is not combined to FG Alum/3D-MPL. Group 4 will allow the evaluation of the immune response induced upon IM Immunization of the combination of the 23 Valent pneumoccocal vaccine and FG Alum/3D-MPL. Groups 5 and 6 constitute a control for the evaluation of the impact of Alum/3D-MPL on FG when it is not combined with the 23 Valent pneumoccocal vaccine. The RSV live immunisations was a control for the immune response induced upon natural RSV IN infection (Groups 7). Finally, group 9 is a control for the impact of Alum/3D-MPL alone.

TABLE 1

Groups	Antigen	Adjuvant	Route
1	none	none	
2	23 Valent Pneumune(11.5μg)	none	IM
3	23 Valent Pneumune (11.5μg)	Alum/3D-MPL	IM
4	23 Valent Pneumune (11.5μg) / FG (2μg)	Alum/3D-MPL	IM
5	FG (2μg)	Alum/3D-MPL	IM
6	FG (2μg)	Alum	IM
7	RSV Live (Lot 14: 10^5PFU)	none	IN
8	none	Alum/3D-MPL	IM
9	23 Valent Pneumune (11.5μg)	none	IP
10	23 Valent Pneumune (11.5μg)	Alum/3D-MPL	IP

2. HUMORAL RESPONSE.

2.1. Anti-FG antibodies

- The analysis of specific anti-FG antibodies at 28 d Post I and 14 d Post II shows the induction of similar antibody responses upon Immunization with FG Alum/3D-MPL / 23-Valent (Group 4) or FG Alum/3D-MPL alone (Group 5) with slightly higher titers observed for FG Alum/3D-MPL / 23-Valent (***Figure 1***). The Immunization with FG Alum (Group 6) induces expectedly lower antibody titers at both time points. Statistical analysis shows that there is indeed a significant difference between the anti-FG Ig titers induced by Groups 4 -6 at 28 d Post I and 14 d Post II.

RESULTS

- The analysis of anti-RSVA neutralizing antibodies (***Figure 2***) presents a parallel profile as the one observed for the anti-FG Ig responses.

- Based on the above results, the anti-FG / anti RSV neutralizing antibody ratio (28d PostII) was calculated and showed that the ratio of FG Alum/3D-MPL / 23-Valent, FG Alum/3D-MPL and FG Alum compares to the ratio induced by the RSV IN group (***Figure 3***).

- An in depth statistical analysis on individual anti-FG Ig, IgG1 and IgG2a isotype titers showed that the addition of the 23-valent vaccine to FG Alum/3D-MPL maintains the Ig and IgG1 responses and increases significantly the IgG2a responses (***Figure 4A***). In addition, the analysis showed that the IgG1/IgG2a ratio and the IgG1 titer of FG Alum/3D-MPL / 23 valent and FG Alum/3D-MPL were similar although significantly different from FG Alum (***Figure 4B***). The three formulations had significant differences in their IgG2a titers.

2.2. Anti-Pneumococcal Polysaccharide IgG

While mice and other rodents may produce IgM against polysaccharide immunogens, they do not normally produce IgG against polysaccharides. This is because their immune system lacks the signals to induce isotype switching to a T-independent antigen such as a polysaccharide. Thus, the measurement of anti-polysaccharide IgG in mice is a sensitive test for the induction of an improved T-independent immune response.

The concentrations of IgG induced against four of the 23 polysaccharides are presented in ***Figure 5***. As we had noted in other experiments, no IgG was produced against polysaccharide types 6B and 23F. However, there was measurable IgG produced against types 14 and 19F.

- Groups that did not contain 23-Valent vaccine did show any detectable IgG to polysaccharides 14 and 19F, confirming the specificity of the measurements.

- A comparison of the route of immunisation reveals that when the 23-valent vaccine is adjuvanted with Alum/3D-MPL, the intra-muscular route appears to be better than inter-peritoneal for type 19F, whereas the reverse is true for type 14. With plain 23-Valent, there does not seem to be a significant difference.

- 23Valent + Alum/3D-MPL induces greater IgG for types 14 and 19F, in both IM and IP immunisation routes when compared to 23-Valent alone. When combined with FG,

however, there is a reduction in the IgG response. Nevertheless, the IgG response to 19F in 23-Valent+FG/Alum/3D-MPL is still greater than that induced by 23-Valent alone (compare groups 3 and 4), and the response to type 14 is improved.

3. CELLULAR RESPONSE.

- The induced FG-specific lymphoproliferation did not show any difference between FG Alum/3D-MPL +/- 23 Valent and FG Alum, both in spleen cells and in lymph node cells (***Figure 6***).

- The analysis of the production of IL-5 and IFN-γ suggests that the mixture of the 23 valent vaccine with FG Alum/3D-MPL does not hamper the production of IFN-γ but could increase somewhat the production of IL-5 (***Figure 7***). This observation is confirmed by a 2 to 8 fold difference in the IFN-γ / IL-5 ratio observed at two doses of FG (10 and 1 μg FG/ml) used for *in vitro* restimulation. However, the FG Alum/3D-MPL / 23-valent vaccine still induces a much higher IFN-γ / IL-5 ratio than FG Alum.

CONCLUSIONS

The analysis of the induced anti-FG Ig specific and anti RSVA neutralizing antibody titers shows that the combination of FG Alum/3D-MPL with the 23 valent pneumococcal vaccine does not hamper the induction of the response observed with FG Alum/3D-MPL alone. The quality of the response measured by the anti-FG/anti-RSVA neutralizing antibody ratio also remains unchanged and close to the ratio observed upon natural infection with RSV true the IN route.

Analysis of the induced FG specific cell mediated response suggest that the addition of the 23 valent vaccine to FG Alum/3D-MPL does not affect the induction of lymphoproliferation in both spleen cells and lymph node cells. Furthermore, the induction of a Th1 type response typically observed with FG Alum/3D-MPL is not hampered by the addition of the 23 valent as measured by in vitro cytokine production of FG specific spleen and lymphnode cells and appears to be enhanced when analysing the FG specific isotype antibody distribution. Indeed, the production of IFN-γ, marker of a Th1 response, remains unchanged upon addition of the 23 valent vaccine to FG Alum/3D-MPL while the production of Il-5, marker of a Th2 response is only slightly increased. Interestingly, the addition of the 23 valent vaccine to FG Alum/3D-MPL significantly increases the production of IgG2a antibodies, marker of a Th1 response, while it is not affecting the IgG1 (marker of a Th2 response) nor the IgG1/IgG2a response.

Thus the combination of FG Alum/3D-MPL and 23-valent vaccine does not affect the response observed with FG Alum/3D-MPL and therefore the advantages linked to the FG Alum/3D-MPL formulation versus FG Alum i.e., induction of high primary and secondary neutralizing antibody responses and a Th1 response as measured by the presence of IgG2a antibodies and the induction by high levels of IFN-γ are maintained.

Combination of 23Valent Pneumune with FG Alum/3D-MPL results in increased IgG production in 2 of 4 polysaccharides tested, but most dramatically for type 19F. 23-Valent alone with Alum/3D-MPL gave the highest IgG concentrations.

In conclusion, there is a favourable combination of 23-Valent pneumococcal vaccine with RSV FG Alum/3D-MPL in which there is a synergistic effect for both vaccines in that both vaccines show improvements in certain immunological tests, and no inhibition in any tests.

77 78

Claims

1. A vaccine composition comprising:

(a) one or more *Streptococcus pneumoniae* polysaccharides either conjugated to a protein or peptide, or non-conjugated; and

(b) an RSV antigen

in conjunction with an adjuvant which is a preferential stimulator of TH1 cell response.

2. A vaccine composition according to claim 1 in which the *Streptococcus pneumoniae* polysaccharide is conjugated preferentially to a protein from the group comprising; Protein D from Haemophilus influenzae B, a lipidated version thereof (lipoprotein D); Tetanus toxin, and Diphtheria Toxin.

3. A vaccine composition according to claim 1 or claim 2 which additionally comprises a carrier.

4. A vaccine composition according to any one of claims 1 to 3 in which the preferential stimulator of TH1-cell response is selected from the group of adjuvants comprising: 3D-MPL, 3D-MPL wherein the size of the particles of 3D-MPL is preferably about or less than 100nm, QS21, a mixture of QS21 and cholesterol, and a CpG oligonucleotide.

5. A vaccine composition according to claim 4 in which the preferential stimulator of TH1-cell response is 3D-MPL.

6. A vaccine composition according to any one of claims 1 to 5 in which the *Streptococcus pneumoniae* polysaccharide is a 23-valent pneumococcal polysaccharide vaccine.

7. A vaccine compostion according to any one of claims 1 to 5 in which the *Streptococcus pneumoniae* polysaccharide is an 11-valent conjugated pneumococcal polysaccharide vaccine.

8. A vaccine composition according to any one of claims 1 to 7 in which the RSV antigen is a human RSV envelope glycoprotein.

9. A vaccine composition according to any one of claims 1 to 8 in which the RSV antigen is chimeric FG or a fragment thereof.

10. A vaccine composition according to any one of claims 1 to 9 in which an influenza virus antigen is additionally present.

11. A vaccine composition according to any one of claims 1 to 10 in which the carrier is selected from the group comprising aluminium hydroxide, aluminium phosphate and tocopherol and an oil in water emulsion.

78

LEGALIZACIONES

OFICINA DE PATENTES

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El suscrito, un funcionario debidamente autorizado conforme a los Artículos 74(1) y (4) de la Ley de Desreglamentación y Contratación de 1994 para firmar y expedir certificados en nombre del Contralor General, certifico que la copia adjunta es una copia correcta de los documentos archivados originalmente con respecto a la solicitud de patente que allí se identifica.

De acuerdo con las Reglamentaciones de Patentes de 1982 (Re-registro de Compañías), si una compañía nombrada en este certificado y en los documentos adjuntos se ha vuelto a registrar según la Ley de Compañías de 1980 con el mismo nombre con el que fue registrada inmediatamente antes del re-registro, salvo la sustitución o inclusión como la última parte del nombre de las palabras "sociedad anónima" o su equivalente en galés, las referencias al nombre de la compañía y sus documentos adjuntos serán tratadas como referencias al nombre con el cual fue re-registrada.

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(FIRMA) ANDREW BREWER FECHA: 7 de abril de 2000

(SELLO DE LACRE)

OFICINA DE PATENTES

Referencia No. **RB/RH/B45180** No. **9909077.1** 20 de abril de 1999

OFICINA DE PATENTES: Solicitud de adjudicación de una Patente.

Forma 1/77 Ley de Patentes de 1977

1. TITULO DEL INVENTO: **"COMPOSICIONES NOVEDOSAS"**

2. INFORMACIÓN DEL SOLICITANTE:

2a. Nombre: SmithKline Beecham Biologicals S.A.

Pais: Bélgica

2b N/A

2c Dirección: Rue de l'Institut 89
B-1330 Rixensart
Bélgica

Código Postal:

PAIS. BELGICA

ADP No. **05781117001**

2d **Segundo Solicitante:**

N/A

3. Ha nombrado Ud. a alguien que le represente en su solicitud? SI

Nombre del Agente: A M R TYRRELL
SmithKline Beecham
Corporate Intellectual Property
Two New Horizions Court,
Brentford, Middlesex

Código postal TW8 9EP

No. ADP

4. Número de Referencia: **FB/RH/B45180**

5. Solicita Ud. que esta solicitud sea tratada como si hubiera sido radicada en la fecha de presentación de una solicitud anterior: NO

6. No aplica

7. Es usted el único inventor o inventor conjunto? NO

8. Cuántas hojas describen esta solicitud? 16 - Reivindicaciones: 2-

Resumen: - - Dibujos - 4 -

9. Solicitud: Solicitamos la adjudicación de una patente teniendo en cuenta esta solicitud.

(FIRMA) A W R TYRRELL Fecha: 19/04/99

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COMPOSICIONES NOVEDOSAS

REIVINDICACIONES:

1. Una composición de vacuna que comprende:

a) uno o más polisacáridos *Streptococcus pneumoniae* conjugados a una proteína o a un péptido, o no conjugados;y

b) un antígeno RSV,

junto con un adyuvante que es un estimulador preferencial de respuesta de la célula TH1.

2. Una composición de vacuna según la reivindicación 1, en la cual el polisacárido *Streptococcus pneumoniae* es conjugado preferencialmente a una proteína del grupo compuesto por: Proteína D de Haemophilus inflenzae B, una versión lipidada de la misma (lipoproteína D); toxina de Tétano, y Toxina de Difteria.

3. Una composición de vacuna según la reivindicación 1 o 2, que adicionalmente comprende un vehículo.

4. Una composición de vacuna según cualquiera de las reivindicaciones 1 a 3, en la cual el estimulador preferencial de la respuesta de la célula TH1 es seleccionado del grupo de adyuvantes compuestos por: 3D-MPL, 3D-MPL, en donde el tamaño de partículas de 3D-MPL es preferiblemente alrededor de o menos de 100 nm, QS21, una mezcla de QS21 y colesterol, y un oligonucleótido CpG.

5. Una composición de vacuna según la reivindicación 4, en la cual el estimulador preferencial de la respuesta de célula TH1 es 3D-MPL.

6. Una composición de vacuna según cualquiera de las reivindicaciones 1 a 5, en la cual el polisacárido *Streptococcus pneumoniae* es una vacuna polisacárida neumococo de 23 valentes.

7. Una composición de vacuna según cualquiera de las reivindicaciones 1 a 5, en la cual el polisacárido *Streptococcus pneumoniae* es una vacuna polisacárida neumococo de 11 valentes.

8. Una composición de vacuna según cualquiera de las reivindicaciones 1 a 7, en la cual el antígeno TSV es una glicoproteina humana RSV envolvente.

9. Una composición de vacuna según cualquiera de las reivindicaciones 1 a 8, en la cual el antígeno RSV es un FG quimérico o un fragmento del mismo.

10. Una composición de vacuna según cualquiera de las reivindicaciones 1 a 9, en la cual un antígeno del virus de influenza está presente adicionalmente.

11. Una composición de vacuna según cualquiera de las reivindicaciones 1 a 10, en la cual el vehículo es seleccionado del grupo compuesto por hidróxido de aluminio, fosfato de aluminio y tocoferol y una emulsión aceite en agua.

B451895

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I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

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Signed P. Mahoney

Dated 6 April 2000

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30 JUN 99 E458175-1 ... P01/7700 0.00 - 9915106.0

28 JUN 1999

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Notes

Please type, or write in dark ink using CAPITAL letters. A prescribed fee is payable for a request for grant of a patent. For details, please contact the Patent Office (telephone 071-483 4700).

Rule 16 of the Patents Rules 1990 is the main rule governing the completion and filing of this form.

❷ Do not give trading styles, for example, 'Trading as XYZ company', nationality or former names, for example, 'formerly (known as ABC Ltd' as these are not required.

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The Patent Office

Request for grant of a Patent

9915106.0

Form 1/77 **Patents Act 1977**

❶ Title of invention

1 Please give the title of the invention VACCINE

❷ Applicant's details

❑ **First or only applicant**

2a If you are applying as a corporate body please give:

Corporate Name SmithKline Beecham Biologicals s.a.

Country *(and State of incorporation, if appropriate)* Belgium

2b If you are applying as an individual or one of a partnership please give in full:

Surname

Forenames

2c **In all cases,** please given the following details:

Address: rue de l'Institut 89, B-1330 Rixensart

UK postcode *(if applicable)* 578117001

Country Belgium

ADP number ~~5800974002~~ *(if known)*

2d, 2e and 2f: If there are further applicants please provide details on a separate sheet of paper

❑ **Second applicant** ***(if any)***

2d If you are applying as a corporate body please give:
Corporate Name

Country *(and State of Incorporation, if appropriate)*

2e If you are applying as an individual or one of a partnership please give in full:

Surname:

Forenames:

2f **In all cases,** please give the following details:

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ADP number
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❸ *An address for service in the United Kingdom must be supplied*

Please mark correct box.

❸ **Address for service details**

3a Have you appointed an agent to deal with your application?

Yes ☒ No ☐ ➲ ***go to 3b***

❶

please give details below

Agent's name MARCUS J W DALTON

Agent's address SmithKline Beecham
Corporate Intellectual Property
New Horizons Court
Great West Road
Brentford, Middlesex

Postcode TW8 9EP

Agent's ADP number 412231300 6

3b: *If you have appointed an agent, all correspondence concerning your application will be sent to the agent's United Kingdom address.*

3b If you have not appointed an agent please give a name and address in the United Kingdom to which all correspondence will be sent:

Name

Address

Postcode

Daytime telephone number *(if available)*

ADP number
(if known)

❹ Reference number

4. Agent's or applicant's reference number *(if applicable)* MJWD/B45185

❺ Claiming an earlier application date

5. Are you claiming that this application be treated as having been filed on the date of filing of an earlier application?

Please mark correct box

Yes ☐ No ☒ ➲ *go to 6*

❶ ***please give details below***

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☐ filing date *(day month year)*

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15(4) (Divisional) ☐ 8(3) ☐ 12(6) ☐ 37(4) ☐

❻ Declaration of priority

❻ If you are declaring priority from a PCT Application please enter 'PCT' as the country and enter the country code (for example, GB) as part of the application number

Please give the date in all number format, for example, 31/05/90 for 31 May 1990

6. If you are declaring priority from previous application(s), please give:

Country of Filing	Priority application number *(if known)*	Filing Date *(day, month, year)*

any applicant is not an inventor
•there is an inventor who is not
•an applicant, or
•any applicant is a corporate body.

❼ Inventorship

7. Are you (the applicant or applicants) the sole inventor or the joint inventors?
Please mark correct box
Yes ☐ No ☒ ➲ *A Statement of Inventorship on Patents form 7/77 will need to be filed (see Rule 15).*.

❽ *Please supply duplicates of claim(s), abstract, description and drawings).*

❽ Checklist

8a Please fill in the number of sheets for each of the following types of document contained in this application

Continuation sheets for this Patents Form 1/77 –

Claim(s) 2 Description 15

Abstract ☐ Drawing(s) ☐

8b Which of the following documents also accompanies the application?

Priority documents *(please state how many)* –

Translation(s) of Priority documents *(please state how many)* –

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Patents Form 9/77 - Preliminary Examination Report –

Patents Form 10/77 - Request for Substantive Examination –

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❾ Request

I/We request the grant of a patent on the basis of this application.

Signed Marcus J W Dalton Date: 28/06/99

MARCUS J W DALTON *(day month year)*
Chartered Patent Attorney
Attorney for the Applicant

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96 88

VACCINE

The present invention relates to new vaccine formulation comprising a Respiratory Syncitial Virus (RSV) antigen and a 'CpG' containing immunostimulating oligonucleotide, methods for preparing it and its use in therapy. In addition the present invention relates to new combination vaccines for administration to children and to the elderly.

Respiratory Syncitial virus (RSV) occurs in seasonal outbreaks, peaking during the winter in temperate climates and during the rainy season in warmer climates. Wherever the area, RSV tends to have a regular and predictable pattern and other respiratory viral pathogens that occur in outbreaks are rarely present concurrently.

RSV infection is almost certainly under-diagnosed 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. Morbidity and mortality may be considerable: pneumonia has been reported in 33 to 67 % of cases, and mortality rates of 5 to 20 % have been reported.

A number of studies have evaluated the contribution of RSV infection to respiratory illness and mortality in nursing homes. Infection rates with RSV in the nursing homes has been reported as 9% in a home in France of which 6% died, 8% in South Carolina, 27% in Rochester of which 5% died and 21% in Los Angeles.

Finally, 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. Dowell identified RSV as one of the four most common pathogens causing severe lower respiratory tract disease resulting in hospitalisation of adults. Falsey 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.

Taken together, 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 both community dwelling healthy and institutionalised elderly.

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

RSV vaccination with whole inactivated virus formulated with alum, led to exacerbation of disease in children subsequently exposed to natural RSV infection. There is therefore a need for a safe and effective vaccine to provide protection against this pathogen.

RSV has two envelope glycoproteins, the F protein having a molecular weight of 68,000 to 70,000 Daltons and a larger G glycoprotein having a molecular weight of 84,000 to 90,000 Daltons (Collins et al J. of Virology Vol 49 pp 572-578 (1984)).

F G fusion proteins, typically comprising the extracellular domain of both proteins, are known (US 5,194,595 Upjohn).

Vaccine preparations comprising FG constructs and 3D-MPL are described in WO 98/18819 (SmithKline Beecham Biologicals s.a.).

Immunomodulatory oligonucleotides contain unmethylated CpG dinucleotides ("CpG") and are known (WO 96/02555, EP 468520). CpG is an abbreviation for cytosine-guanosine dinucleotide motifs present in nucleic acid. Historically, it was observed that the DNA fraction of BCG could exert an anti-tumour effect. In further studies, synthetic oligonucleotides derived from BCG gene sequences were shown to be capable of inducing immunostimulatory effects (both in vitro and in vivo). The authors of these studies concluded that certain palindromic sequences, including a central CG motif, carried this activity. The central role of the CG motif in immunostimulation was later elucidated in a publication by Krieg, Nature 374, p546 1995. Detailed analysis has shown that the CG motif has to be in a certain sequence context, and that such sequences are common in bacterial DNA but are rare in vertebrate DNA.

It is currently believed that this evolutionary difference allows the vertebrate immune system to detect the presence of bacterial DNA (as occurring during an infection) leading consequently to the stimulation of the immune system. The immunostimulatory sequence as defined by Krieg is:

Purine Purine CG pyrimidine pyrimidine and where the CG motif is not methylated. In certain combinations of the six nucleotides a palindromic sequence is present. Several of these motifs, either as repeats of one motif or a combination of different motifs, can be present in the same oligonucleotide. The presence of one or more of these immunostimulatory sequence containing oligonucleotides can activate various immune subsets, including natural killer cells (which produce interferon γ and have cytolytic activity) and macrophages (Wooldrige et al Vol 89 (no. 8), 1977). Although other unmethylated CpG containing sequences not having this consensus sequence have now been shown to be immunomodulatory.

The present invention provides a vaccine preparation comprising an immunostimulatory CpG oligonucleotide and a RSV antigen. Preferably the RSV antigen is a RSV envelope glycoprotein or derivative thereof derived from, preferably, strain A. More preferably the antigen is selected from F glycoprotein, G glycoprotein or a FG fusion protein or immunogenic derivatives thereof.

Typically immunogenic derivatives include wherein the protein is devoid of the transmembrane domain ie F Δtm, G Δtm and F Δtm GΔtm. Preferably the

signal sequence is deleted from the G protein. It is preferred that at least 80% (contiguous sequence) of the extracellular domain of the F or G protein is present. A preferred example is 1-526 or 1-489 amino acid of the F protein. Other preferred embodiments include amino acid 69-298 or alternative positions 97-279 of the G protein, and $F_{1\text{-}526}G_{69\text{-}298}$ fusion protein. An alternative fusion comprises 1-489 amino acid from F followed by 97-279 of G protein.

The preferred oligonucleotides preferably contain two or more CpG motifs separated by six or more nucleotides. The oligonucleotides of the present invention are typically between 15-45 oligonucleotides in length and 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 including oligonucleotides with mixed internucleotide linkages.

Preferred oligonucleotides have the following sequences: The sequences preferably contain all phosphorothioate modified internucleotide linkages.

WD000 1: TCC ATG ACG TTC CTG ACG TT

WD000 2: TCT CCC AGC GTG CGC CAT

WD000 3: ACC GAT AAC GTT GCC GGT GAC G

WD000 7: ACC GAT GAC GTC GCC GGT GAC GGC ACC ACG

The CpG oligonucleotides utilised in the present invention may be synthesized by any method known in the art (eg EP 468520). Conveniently, such oligonucleotides may be synthesized utilising an automated synthesizer. Methods for producing phosphorothioate oligonucleotides or phosphorodithioate are described in US5,666,153, US5,278,302 and WO95/26204.

When CpG and aluminium salt, such as aluminium hydroxide or aluminium phosphate (Alum) are both present this synergistically enhances anti RSV antigen specific antibody. In particular, such a combination adjuvant significantly enhances the levels of IgG2a antibody a marker of a TH1 response. Moreover, the adjuvant combination of a CpG oligonucleotide and an aluminium salt allows a cell mediated response as determined by specific lymphoproliferation.

Accordingly, in one embodiment of the invention there is provided a vaccine formulation comprising a RSV antigen and an immunostimulatory CpG

oligonucleotide in combination with an aluminium salt. Preferably the aluminium salt is aluminium hydroxide.

Other vaccine excipients may be added to the formulation for the invention. Preferred additional immunostimulants include for example saponin adjuvants, such as QS21.

In the vaccine of the present invention, an aqueous solution of the protein(s) can be used directly for mixing with the adjuvant. Alternatively, the protein can be lyophilised.

The antigens of the present invention may be expressed in any suitable host, such as bacterial, mammalian, insect, yeast and fungal cells. The use of insect cells such as Sf9 cells is described by Du et al, BIO/TECHNOLOGY 12,1994, 813-818. Preferably the proteins of the invention are expressed in insect cells using a recombinant baculovirus (Wathen et al J.Gen Virol 1989, 70 pp2625-2635). Most preferably however the proteins of the invention are produced in eukaryotic cells, particularly in Chinese Hamster Ovary (CHO) cells and Vero cells and purified by the method as disclosed in WO98/18819 (SmithKline Beecham Biologicals s.a.).

In a preferred embodiment of the invention the antigen is produced by expression in mammalian cells from a DNA sequence having optimised mammalian codon usage. Optimisation of the codon usage involves the replacement of at least one non-preferred or less preferred codon in a natural gene encoding a protein by a preferred codon encoding the same amino acid. Mammalian genes expressed at high levels typically have C or G at their degenerative position (third base in the codon) whereas the RSV or more generally paramyxoviridae codons have A or T. At least one codon, and more prefereably all the codons of the RSV protein can be changed to best fit mammalian cell usage, that is, the one (or ones) that is the most prevalent as shown below.

Ala: GCC	Cys: TGC	His: CAC	Met: ATG	Thr: ACC
Arg: CGC AGG CGG	Gln: CAG	Ile: ATC	Phe: TTC	Trp: TGG
Asn: AAC	Glu: GAG	Leu: CTG	Pro: CCC	Tyr: TAC
Asp: GAC	Gly: GGC	Lys: AAG	Ser: AGC TCC	Val: GTG

Each amino acid encoded by one of these codons are then considered optimised.

The invention also provides DNA encoding such a protein or immunogenic derivative thereof in which the codon usage of one or more nucleic acids has been substantially optimised and a process for expressing said DNA in a CHO or insect cell.

The antigens may also be made using a recombinant live microorganism, such as a virus or bacterium as the expression system. The gene of interest can be inserted into the genome of a live recombinant virus or bacterium. Inoculation and *in vivo* infection with this live vector will lead to *in vivo* expression of the antigen and induction of immune responses. Viruses and bacteria used for this purpose are for instance: poxviruses (e.g; vaccinia, fowlpox, canarypox), alphaviruses (Sindbis virus, Semliki Forest Virus, Venezuelian Equine Encephalitis Virus), adenoviruses, adeno-associated virus, picornaviruses (poliovirus, rhinovirus), herpesviruses (varicella zoster virus, etc), Listeria, Salmonella , Shigella, BCG. These viruses and bacteria can be virulent, or attenuated in various ways in order to obtain live vaccines. Such live vaccines when formulated with a CpG oligo nucleotides also form part of the invention.

The invention further relates to methods for constructing and expressing the proteins of the invention and methods to optimise the codon usage of the nucleic acid sequences which encode such proteins.

89 94

The anitgens of the present invention may also be presented as a nucleic acid encoding said antigen and formulated with a CpG oligonucleotide.

Vaccine preparation is generally described in New Trends and Developments in Vaccines, edited by Voller et al, University Park Press, Baltimore, Maryland, USA 1978 and in Vaccine Design: The Subunit and Adjuvant Approach, edited by Powell and Newman, Plenum Press, New York, 1995. Encapsulation within liposomes is described, for example, by Fullerton, US Patent 4,235,877. Conjugation of proteins to macromolecules is disclosed, for example by Likhite, US Patent 4,372,945 and by Armor et al, US Patent 4,474,757.

The amount of protein in each vaccine does 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. Generally, it is expected that each dose will comprise 1-1000 μg of protein, preferably 2-100 μg, most preferably 5-50 μg. An optimal amount for a particular vaccine can be ascertained by standard studies involving observation of appropriate immune responses in subjects. Following an initial vaccination, subjects may receive one or several booster immunisations adequately spaced.

Suitably the CpG will be present in the range 100μg per dose to 3000μg, preferably 250-750μg, such as 500μg per dose.

Suitably the vaccine used in the present invention may comprise a carrier such as an aluminium salt, eg aluminium hydroxide ($A1(OH_3)$), aluminium phosphate or aluminium phosphate sulfate (alum), or a non-toxic oil in water emulsion or a mixture thereof.

If an aluminium salt (preferably aluminium hydroxide) is used as a carrier it is generally present in the range of 50 to 100μg (human: 500 to 1000μg) preferably 500μg per dose.

Non-toxic oil in water emulsions preferably contain a non-toxic oil, eg squalene and an emulsifier such as polysorbitan monoleate (Tween 80), in an aqueous carrier such as phosphate buffered saline.

If desired the vaccine used in the present invention may comprise an additional adjuvant, preferably a saponin adjuvant such as QS21 as described for example in WO 9517210, optionally in the presence of a sterol, such as cholesterol as described for example in PCT/EP96/01464.

If desired, other antigens may be added, in any convenient order, to provide multivalent vaccine compositions as described herebelow.

In a preferred aspect the vaccine formulation of the invention additionally comprises a Streptococcus pneumoniae antigen.

Streptococcus pneumoniae is a gram positive bacteria responsible for considerable morbidity and mortality, particularly in the young and aged. Expansive colonisation of the respiratory tract, and middle ear, especially in young children, is the single most common cause for hospital visits in the US. The bacteria may become invasive, infecting the lower lungs and causing pneumonia. The rate of pneumococcal pneumonia in the US for persons over 60 years of age is estimated to be 3 to 8 per 100,000. In 20% of cases this leads to bacteremia, and other manifestations such as meningitis, with a mortality rate close to 30% even with antibiotic treatment. There are 90 known serotypes of *Streptococcus pneumoniae* which are determined by the structures of the capsular polysaccharide surrounding the bacteria, and this is its major virulence factor.

A 17 - valent pneumococcal vaccine (Moniarix) is known, based on the purified polysaccharides of the pneumococcal serotypes most commonly involved in invasive disease. The method of purification of these polysaccharides was disclosed in European Patent 72513 B1. Vaccine efficacy trials with lower valent vaccines demonstrated a 70 to 90% efficacy with respect to serotypes present in the combination. Case controlled studies in the US in persons >55 years using a 14 valent vaccine demonstrated 70% efficacy (Mills and Rhoads 1996). Inclusion of additional polysaccharides (to make a 23-valent pneumococcal vaccine) were accepted on the basis of an adequate serological response, even though there was clinical efficacy data lacking (Brown 1995).

Pneumococcal polysaccharides can be rendered more immunogenic by chemically coupling them to protein carriers, and clinical efficacy trials are being performed to verify this concept for efficacy in preventing infant Otitis media.

There are two conjugation methods generally used for producing immunogenic polysaccharide constructs: (1) direct conjugation of carbohydrate and protein; and (2) indirect conjugation of carbohydrates and protein via a bifunctional linker or spacer reagent. Generally, both direct and indirect conjugation require chemical activation of the carbohydrate moiety prior to derivatisation. See for example US 5,651,971 and Dick & Beurret, "Glycoconjugates of Bacterial Carbohydrate Antigens," Conjugate Vaccines, J.M. Cruse & R.E. Lewis (eds), Vol. 10, 48 - 114 (1989).

In another preferred aspect the vaccine composition of the invention additionally comprises a Group B Streptococcus antigen.

Among the infants, Group B streptococci (GBS) are a main cause of life threatening bacterial infections, e.g. pneumonia and meningitidis. Additionally GBS has emerged as an important pathogen in adults, more especially the elderly patients or patients with chronic underlying diseases.

All strains of GBS express a polysaccharide capsule. Among the nine capsular serotypes identified, strains of the four classical serotypes (Ia, Ib, II and III) are responsible for most invasive neonatal infections. Approximately 90% of these strains express either Rib or alpha, two members of the same family of streptococcal cell surface proteins, and have been shown to confer protective immunity against GBS in animal models.

Optionally the vaccine composition of the invention additionally comprises one or more of a number of other antigens such as an antigen against *influenza* virus or PIV-3.

In order that this invention may be better understood, the following examples are set forth. These examples are for purposes of illustration only, and are not to be construed as limiting the scope of the invention in any manner.

EXAMPLES

1. Formulations:

FG antigen was expressed in CHO cells and purified according to WO98/18819.

1.1. CpG or MPL based formulations

When needed, $Al(OH)_3$ was diluted in H_2O before adsorption of the FG (2 μg) antigen for 30 minutes. Subsequently MPL or CpG was added and incubated for thirty minutes. The formulations were then buffered with 10 fold concentrated PO_4-NaCl pH 6.8. Thiomersal at 1 mg/ml or phenoxy at 5mg/ml was added as preservative.

All incubations were carried out at room temperature with agitation. The formulations were prepared simultaneously for the 2 injections with a 12-day maturation of the finalized formulations before the first injection.

1.2. Composition of formulation constituents:

COMPONENT	CONCENTRATION (μG/ML)	BUFFER
FG	484	PO4/NaCl 10/150mM pH 6.8
MPL	1019	H_2O
CpG	5000	H_2O
$AlPO_4$	1000	NaCl 150mM pH6.1
$Al(OH)_3$	10380	H_2O

2. Immunisation protocol:

9 groups of 10 mice were immunized by different routes (50 μl) at days 0 and 28 with various formulations (see ***Table I***). Groups 8 was immunized with live RSV by the intra-nasal route (60μl). Sera were obtained at days 28 (28 d Post I) and 42 (14 d Post II). On day 42, spleen cells were taken from 5 mice of all groups for CMI analysis.

91 A2
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3. Humoral response:

3.1. Anti-FG antibodies:

All humoral results were performed for 10 mice/ group for the anti-FG titers (individual responses except for groups 6, 7 and 9 and cellular results were presented for 5 mice/group.

Individual sera were obtained 28 days after the first immunization and 14 days after the second immunisation and were tested for the presence of FG specific Ig antibodies and their isotype (IgG2a, IgG1) distribution.

The assay protocol was as follows: coating overnight at 4°C with 50 µl of purified FG 54/023 (1µg/ml) per well, saturation 1h at 37°C, incubation with sera 1h30 at 37°C, incubation with anti-mouse Ig biotin 1/1500 (or IgG1, IgG2a biotin 1/1000) 1h30 at 37°C, incubation with strepta-peroxydase 1/2500 30 min at 37°C, incubation with OPDA Sigma 15 min at RT, stop with H_2SO_4 2N.

OD were monitored at 490 nm and the titers determined by linear regression (y=a.logx + b): titer = serum dilution giving 50% reduction of the maximal OD. 95% confidence limits (IC 95%) were calculated for each group.

Individual sera obtained 14d Post II were tested for the presence of neutralising antibodies using the following protocol: 50 µl of serial two-fold dilutions of sera (first dilution 1/250) were incubated for 1 hour at 37°C with 50 µl of a mixture containing 500 pfu of RSV-A/Long (Lot 14) and guinea pig complement (1/25 dilution) in a 96 well plate in duplicate. 100 µl of a HEp-2 cell suspension at 10^5 cells/ml were then added to each well and the plates were incubated for 4 days at 37°C in the presence of 5% CO_2.

The supernatants were then aspirated, and after addition of a 100 µl of a WST-1 preparation (dilution 1/12.5) the plates were further incubated for 24H at 37°C in

the presence of 5% CO_2. The OD were monitored at 595 nm and the titers determined by linear regression (y=a.logx + b): titer = serum dilution giving 50% reduction of the maximal OD observed for the uninfected cells.

Controls in test included a pool of randomly chosen human sera (Human pool) and Sandoglobuline (lot 069, generic human IgG produced by Sandoz).

4. Cellular response

Spleen cells were isolated 14d Post II from groups 1-8 and from naïve mice (Group 9) for use as a negative control for the FG-specific cellular response analysis. Samples were analyzed for both FG-specific lymphoproliferation and cytokine (IFN-? + IL-5) secretion.

Proliferation was evaluated after a 96h incubation of $4x10^5$ cells/well of 96 well plates with 200 μl of media containing 10 to 0.03 μg/ml of FG (3-fold dilutions). Upon ^{3}H-thymidine incorporation, the FG specific proliferation was measured following our standard protocol.

Cytokine induction was evaluated after 96 h incubation of $2.5x10^6$ cells per well of 24 well with 1 ml of media containing 10μg to 0.01μg of FG (10-fold dilutions). Supernatants were then harvested to determine the quantity of IFN-γ and IL-5 induced by ELISA following our standard protocol.

RESULTS

1. Groups:

Nine groups received two immunisations of various formulations containing FG formulated with alum 3D-MPL CpG or CpG-Alum. Group 9 constitutes the negative control for the CMI studies. Groups 6 and 7 constitute controls for the

immunogenicity that could be induced by immunization of the mice with the adjuvants alone. The RSV live immunizations was a control for the immune response induced upon natural RSV IN infection (Group 8).

2. Humoral response:

2.1. Anti-FG antibodies

Analysis of the specific anti-FG Ig antibodies at 14 d Post II vaccination was performed. The results show (table 2) that a clear synergistic effect is observed by combining CpG with Alum as FG formulated with CpG-Alum induces twice as many anti-FG-specific Ig antibodies as FG formulated with each of the adjuvants alone. The results also show that alum CpG and alum 3D-MPL induce equivalent levels of antibody.

The analysis of the isotype profiles shows that CpG-Alum induce a IgG1:Ig2a <1 ratio while the three other formulations induce a >1 ratio. CpG and CpG-Alum induce similar IgG1 titers which are two to three times (CpG < CpG-Alum < SB AS1i) lower than those induced by alum 3D-MPL and Alum. CpG-Alum induce high IgG2a titers which are three to five times higher than those induced by CpG and alum 3D-MPL and thirty to fifty times higher than those induced by FG Alum. The addition of Alum to CpG thus not seem to affect the IgG1 titers but rather increases the IgG2a titers by three fold as compared to FG CpG allowing the IgG1/IgG2a ratio to go from >1 to <1.

3. Cellular response:

The induced FG-specific lymphoproliferation shows the induction of equivalent stimulation indexes for CpG alum, 3D-MPL alum and alum except for FG CpG. FG CpG similarly to the adjuvants alone groups and the naïve mice control group,

does not induce any detectable FG specific lymphoproliferation. As observed in other experiments, RSV live IN does not induce a sufficiently high immune response for the lymphoproliferation assay to be able to pick it up.

DISCUSSION AND CONCLUSIONS

The antibody analysis shows a clear linear synergistic effect is observed upon formulating FG with CpG and Alum as compared to FG formulated with either of the adjuvants.

Based on the isotype analysis FG CpG-Alum, FG CpG and FG alum MPL all induce a mixed Th profile as measured by the induction of both IgG2a and IgG1 antibodies. FG Alum however almost exclusively induces IgG1 antibodies, marker of a Th2 response. Based on the level of induced FG-specific IgG2a antibodies the results suggest FG CpG-Alum predominantly induce Th1 antibody isotypes while FG CpG and to a greater extend FG alum 3D-MPL predominantly induce Th2 antibody isotypes. The formulation of FG with both CpG and Alum leads to a three-fold increase in IgG2a titer as compared to FG CpG alone.

Analysis of the induced FG specific cell mediated response suggests that FG CpG does not induce detectable lymphoproliferation. However, similarly to what is observed for the FG-specific Ig antibody responses the formulation of FG with both CpG and Alum does allow the induction of a specific lymphoproliferation.

93 AD 95

TABLE 1

Groups	Antigen	Adjuvant	Route
2	FG (2µg)	CpG	IM
3	FG (2µg)	CpG Alum	IM
4	FG (2µg)	Alum MPL	IM
5	FG (2µg)	Alum	IM
6	/	Control adjuvant	IM
7	/	CpG (100 µg)	IM
8	RSV Live (Lot 14: 10^5PFU)		IN
9	none	none	

TABLE 2

Vaccine Candidate	Group No.	14 days Post II analysis of the immunised Balb/c mice serum (GMT)						
		Anti-FG ELISA Ig	RSV-A Neutra	ELIS / Neutra ratio	IgG1 / IgG2a Ratio	IgG1	Ig G2a	IgG2b
FG CpG	2	66777			1.97	60139	30604	5121
FG CpG-alum	3	122236			0.71	78557	110488	15567
FG SB alum 3DMPL	4	1608022			7.19	178569	24846	17771
FG Alum	5	91568			66.55	161254	2423	617
RSV live	8	4623			0.82	1396	1705	823

Claims

1. A vaccine formulation comprising a RSV antigen and an immunostimulatory CpG oligonucleotide.
2. A vaccine formulation as claimed in claim 1 wherein the RSV antigens are selected from the group, F protein or immunogenic derivative thereof, G protein or immunogenic derivative thereof, or a FG fusion protein or an immunogenic derivative thereof.
3. A vaccine as claimed in claim 1 or 2 wherein the derivative is an antigen essentially devoid of a transmembrane domain.
4. A vaccine as claimed in claims 1 to 3 wherein the derivative is an antigen in which at least one non-preferred or less preferred codon has been replaced by a preferred codon encoding the same amino acid
5. A vaccine formulation as claimed herein additionally comprising an aluminium salt or a saponin adjuvant.
6. A vaccine as claimed herein wherein the oligonucleotide comprises two CpG dincucleotides.
7. A vaccine as claimed herein wherein the CpG oligonucleotide is between 15-45 nucleotides in length.
8. A vaccine as claimed herein wherein the CpG oligonucleotide comprises at least one phosphorothioate internucleotide bond.
9. A vaccine as claimed herein wherein the oligonucleotide is selected from the group:

   WD000 1: TCC ATG ACG TTC CTG ACG TT

   WD000 2: TCT CCC AGC GTG CGC CAT

   WD000 3: ACC GAT AAC GTT GCC GGT GAC G

   WD000 7: ACC GAT GAC GTC GCC GGT GAC GGC ACC ACG
10. A vaccine formulation as claimed in claim 1 to 9 in which a GroupB Streptococcus antigen is additionally present
11. A vaccine formulation as claimed in claim 1 to 10 in which a Influenza virus antigen is additionally present

94 95

12. A vaccine formulation as claimed in claim 1 to 11 in which a Streptococcus pneumoniae antigen is additionally present

13. A vaccine formulation as claimed 1 to 12 in which a PIV-3 antigen is additionally present.

14. A method for the prevention or amelioration of RSV infection of a patient, comprising administering an effective amount of a vaccine of claim 1 to 13 to patient.

15. A vaccine as claimed herein for use as a medicament.

16. A method of producing a vaccine as claimed in any of claim 1 to 8 comprising admixing a RSV antigen and a CpG immunostimulatory oligonucleotide.

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97

LEGALIZACIONES

OFICINA DE PATENTES

Concept House
Cardiff Road
Newport
South Wales
NP10 8QQ

El suscrito, un funcionario debidamente autorizado conforme a los Artículos 74(1) y (4) de la Ley de Desreglamentación y Contratación de 1994 para firmar y expedir certificados en nombre del Contralor General, certifico que la copia adjunta es una copia correcta de los documentos archivados originalmente con respecto a la solicitud de patente que allí se identifica.

De acuerdo con las Reglamentaciones de Patentes de 1982 (Re-registro de Compañías), si una compañía nombrada en este certificado y en los documentos adjuntos se ha vuelto a registrar según la Ley de Compañías de 1980 con el mismo nombre con el que fue registrada inmediatamente antes del re-registro, salvo la sustitución o inclusión como la última parte del nombre de las palabras "sociedad anónima" o su equivalente en galés, las referencias al nombre de la compañía y sus documentos adjuntos serán tratadas como referencias al nombre con el cual fue re-registrada.

De acuerdo con las reglas, las palabras "sociedad anónima" (o en inglés *public limited company)* pueden ser reemplazadas por las letras p.l.c, plc. P.L.C ó PLC.

El re-registro bajo la Ley de Compañías no constituye una nueva entidad legal sino simplemente somete a la compañía a ciertas normas de ley adicionales.

(FIRMA) R. MAHONEY FECHA: 6 de abril de 2000

(SELLO DE LACRE)

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OFICINA DE PATENTES

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Referencia No. **MJWD/B45185** No. **9915106.0** 28 de junio de 1999

OFICINA DE PATENTES: Solicitud de adjudicación de una Patente.

Forma 1/77 Ley de Patentes de 1977

1. TITULO DEL INVENTO: **"VACUNA"**

2. INFORMACIÓN DEL SOLICITANTE:

2a. Nombre: SmithKline Beecham Biologicals S.A.

Pais: Bélgica

2b N/A

2c Dirección: Rue de l'Institut 89
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ADP No. **05781117001**

2d **Segundo Solicitante:**

N/A

3. Ha nombrado Ud. a alguien que le represente en su solicitud? SI

Nombre del Agente: MARCUS J W DALTON
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No. ADP 4122313006

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99

4. Número de Referencia: **MJWD/B45185**

5. Solicita Ud. que esta solicitud sea tratada como si hubiera sido radicada en la fecha de presentación de una solicitud anterior: NO

6. No aplica

7. Es usted el único inventor o inventor conjunto? NO

8. Cuántas hojas describen esta solicitud? 15 - Reivindicaciones: 2-

Resumen: - - Dibujos - 4 -

9. Solicitud: Solicitamos la adjudicación de una patente teniendo en cuenta esta solicitud.

(FIRMA) MARCUS J W DALTON Fecha: 28/06/99

Abogado de Patentes

Favor devolver la forma diligenciada y sus anexos y duplicados, junto con su valor a:

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VACUNA

REIVINDICACIONES:

1. Una formulación de vacuna que comprende un antígeno RSV y un oligonucleótido inmunoestimulatorio.

2. Una formulación de vacuna según la reivindicación 1, en donde los antígenos RSV son seleccionados del grupo proteína F o un derivado inmunogénico de la misma, proteína G o un derivado inmunogénico de la misma, una proteína de fusión FG o un derivado inmunogénico de la misma.

3. Una vacuna según la reivindicación 1 ó 2, en donde el derivado es un antígeno esencialmente desprovisto de un dominio de transmembrana.

4. Una vacuna según las reivindicaciones 1 a 3, en donde el derivado es un antígeno en el cual al menos un codón no preferido o menos preferido ha sido reemplazado por n codón preferido que codifica el mismo amino ácido.

5. Una formulación de vacuna según se reivindica arriba, que adicionalmente comprende una sal alumínica o un adyuvante de saponina.

6. Una vacuna según se reivindica arriba, en donde el oligonucleótido comprende dos dinucleótidos CpG.

7. Una vacuna según se reivindica arriba, en donde el oligonucleótido tiene entre 15 y 45 nucleótidos de longitud.

8. Una vacuna según se reivindica arriba, en donde el oligonucleótido CpG comprende al menos un enlace internucleótido de fosforotioato.

9. Una vacuna según se reivindica arriba en donde el oligonucleótido es seleccionado del grupo:

WD000 1: TCC ATG ACG TTC CTG ACG TT

WD000 2: TCT CCC AGC GTG CGC CAT

WD000 3: ACC GAT AAC GTT GCC GGT GAC G

WD000 7: ACC GAT GAC GTC GCC GGT GAC GGC ACC ACG

10. Una formulación de vacuna según las reivindicaciones 1 a 9, en la cual un antígeno *Streptococcus* del Grupo B está adicionalmente presente.

11. Una formulación de vacuna según las reivindicaciones 1 a 10, en la cual un antígeno del virus de Influenza está adicionalmente presente.

12. Una formulación de vacuna según las reivindicaciones 1 a 11, en la cual un antígeno de *Streptococcus pneumoniae* está adicionalmente presente.

13. Una formulación de vacuna según las reivindicaciones 1 a 12, en la cual un antígeno PIV-3 está adicionalmente presente.

14. Un método para la prevención o alivio de infección RSV de un paciente que consiste en administrar al paciente una cantidad efectiva de una vacuna de las reivindicacinoes 1 a 13.

15. Una vacuna según se reivindica arriba para uso como medicamento.

16. Un método para producir una vacuna según cualquiera de las reivindicaciones 1 a 8, que consiste en mezclar un antígeno RSV y un oligonucleótido inmunoestimulatorio CpG.