PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : A61K 39/00 — A2

(11) International Publication Number: **WO 00/62802**

(43) International Publication Date: 26 October 2000 (26.10.00)

(21) International Application Number: PCT/EP00/03516

(22) International Filing Date: 17 April 2000 (17.04.00)

(30) Priority Data:

9909077.1	20 April 1999 (20.04.99)	GB
9915106.0	28 June 1999 (28.06.99)	GB

(71) Applicant *(for all designated States except US)*: SMITHKLINE BEECHAM BIOLOGICALS SA [BE/BE]; Rue de l'Institut 89, B–1330 Rixensart (BE).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: DESCHAMPS, Marguerite [BE/BE]; SmithKline Beecham Biologicals SA, Rue de l'Institut 89, B–1330 Rixensart (BE).

(74) Agent: PRIVETT, Kathryn, Louise; Corporate Intellectual Property, SmithKline Beecham, Two New Horizons Court, Brentford, Middlesex TW8 9EP (GB).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

Without international search report and to be republished upon receipt of that report.

(54) Title: VACCINE

(57) Abstract

The invention relates to a vaccine formulation comprising a Respiratory Syncytial Virus (RSV) antigen and an immunostimulatory CpG oligonucleotide, to methods of preparing the vaccine formulation and to its use in medicine. Further antigens may be included to provide new combination vaccines for administration to children, to adults and to the elderly.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

VACCINE

The present invention relates to new vaccine formulations comprising a Respiratory Syncytial 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, to adults and to the elderly.

Human Respiratory Syncytial Virus (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 is an enveloped virus with a non-segmented, negative strand ribonucleic acid (RNA) genome of 15,222 nucleotides that codes for 10 messenger RNAs, each coding for a single polypeptide. Three of the ten proteins are transmembrane surface proteins: the G (attachment), F (fusion) and SH proteins. Two proteins are virion matrix proteins (M and M2), three proteins are components of the nucleocapsid (N, P and L), and two proteins are nonstructural (NS1 and NS2). Two antigenically distinct strains of RSV exist, designated strain A and B. Characterization of strains from these groups has determined that the major differences reside on the G proteins, while the F proteins are conserved.

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 is a major cause of serious lower respiratory tract disease in children. It is estimated that 40-50% of children hospitalized with bronchiolitis and 25% of children hospitalized with pneumonia are hospitalized 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 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. RSV vaccination in the 1960s with whole formalin 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)).

FG 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. Alternatively the RSV antigen may be for example inactivated virus.

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 about 50% or at least about 80% (contiguous sequence) of the extracellular domain of the F or G protein is present. Particular examples include 1-526 or 1-489 amino acid of the F protein, 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 as described in 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 preferably 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 is then considered optimised.

The antigen according to the invention may be expressed as a fusion protein. For example, the antigen may be a heterochimeric fusion of an RSV envelope

antigen or an immunogenic derivative thereof with an antigen from a different pathogen. Particular examples include fusions with envelope antigens or immunogenic derivatives from other Paramyxoviruses eg parainfluenza viruses (PIV-1, 2 and 3) or mumps virus or measles virus.

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.

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

The antigens of the present invention are not necessarily recombinant subunit anitigens. The RSV antigen may for example be in the form of inactivated whole virus. Such inactivated virus may be produced from RSV which has been attenuated eg by passaging or by genetic manipulation. A recombinant RSV may be used which has been engineered to contain an envelope antigen from a different strain of RSV eg an RSV strain A backbone with an RSV B envelope protein, optionally attenuated.

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 dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccinees. Such amount will vary depending upon which specific immunogen is employed and how it is presented. 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 ($Al(OH_3)$), 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 95/17210, 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).

Typically the *Streptococcus pneumoniae* component in a vaccine of the present invention will comprise polysaccharide antigens (preferably conjugated), wherein the polysaccharides are derived from at least four serotypes of pneumococcus. Preferably the four serotypes include 6B, 14, 19F and 23F. More preferably, at least 7 serotypes are included in the composition, for example those derived from serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F. More preferably still, at least 11 serotypes are included in the composition, for example the composition in one embodiment includes capsular polysaccharides derived from serotypes 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F (preferably conjugated). In another preferred embodiment of the invention at least 13 polysaccharide antigens (preferably conjugated) are included, although further polysaccharide antigens, for example 23 valent (such as serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F), are also contemplated by the invention. For elderly vaccination (for instance for the prevention of pneumonia) it is advantageous to include serotypes 8 and 12F (and most preferably 15 and 22 as well) with the 11 valent antigenic composition described above to form a 15 valent vaccine, whereas for infants or toddlers (where otitis media is of more concern) serotypes 6A and 19A are advantageously included to form a 13 valent vaccine.

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, eg 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

EXAMPLE 1 - Studies on RSV formulated with CpG containing oligonucleotides

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 1***). 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.

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 Softmaxpro (4 parameters equation) referring to a standard curve and expressed in EU/ml.

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 ($y=a.\log x + b$): 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-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 ^3H-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.

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

EXAMPLE 2 - Studies on RSV formulated with CpG containing oligonucleotides, together with additional antigens (polysaccharide from *Streptococcus pneumoniae*)

1. Formulation process:

Formulations were prepared 4 days before the first injection.

1.1 Al(OH)3-based formulations

CpG (50,10 or 2 μg) was preadsorbed on Al(OH)3 as concentrated monobulk by mixing the immunostimulants with the Aluminium salt one day before the final formulation. Non-adsorbed 11-valent SP, conjugated to protein D, was prepared by mixing the 11 conjugated components by numeric order and adjusting the concentration to 2μg/val/ml with NaCl 150mM. The final formulations were prepared by mixing H_2O and Aluminium salt if needed and preadsorbed CpG. After 5 minutes of incubation non-adsorbed 11-valent SP (0.1μg/val) and /or FG (2μg)

(FG as for Example 1) were added and incubated for 30min. PO_4/NaCl buffer was then added and incubated for 5min before addition of thiomersal (1μg/ml) which was allowed to incubate for 30min.

1.2 Formulations without $Al(OH)_3$

The non-adsorbed 11-valent polysaccharide (PS) was prepared by mixing the 11 valences by numeric order and adjusting the concentration at 2μg/val/ml with NaCl 150mM. The final formulations were prepared by mixing H_2O and PO_4/NaCl buffer and Non-adsorbed 11-valent (0.1μg/val) and /or FG (2μg). After 5 minutes of incubation CpG was added and incubated for 30min. Thiomersal (1μg/ml) was then added and allowed to incubate for 30min.

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 finalized formulations before the first injection.

2. Composition of formulation constituents:

TABLE 3

COMPONENT	AG OR IMST CONC (μG/ML)	AL+++ CONC (μG/ML)	BUFFER
FG	484		PO_4/NaCl 10/150mM pH 6.8
Clinical 11-valent nonadsorbed			
PS1	144		NaCl 150mM pH6.1
PS3	149		NaCl 150mM pH6.1
PS4	114		NaCl 150mM pH6.1
PS5	135		NaCl 150mM pH6.1
PS6b	200		NaCl 150mM pH6.1
PS7	168		NaCl 150mM pH6.1
PS9	175		NaCl 150mM pH6.1
PS14	180		NaCl 150mM pH6.1
PS18	137		NaCl 150mM pH6.1
PS19	140		NaCl 150mM pH6.1
PS23	158		NaCl 150mM pH6.1
QS21	2000		H_2O
CpG1826	20000		H_2O
$Al(OH)_3$		10380	H_2O

2. Immunization protocol:

19 groups of 10 mice were immunized by the intramuscular route (100 μl) at days 0 and 28 with various formulations (see ***Table 4***). Sera were obtained at days 42 and 43 (14/15 d Post II). On day 43, spleen cells were taken from 5 mice of the groups immunized with FG containing formulations as well as from 5 naïve Balb/c mice (group 24, not immunized).

3. Humoral response.

3.1. Anti-FG antibodies:

All humoral results were performed for 10 mice/group (individual response for the anti-FG Ig, IgG1 and IgG2a and neutralization titers) and cellular results were presented for 5 mice/group on pool.

Individual sera were obtained 14/15 days after the second immunization and were tested for the presence of FG specific Ig antibodies and their isotype (IgG2a, IgG1) distribution.

Assays were performed as described in Example 1.

3.2. Anti-Pneumococcal Polysaccharide IgG:

Murine IgG to pneumococcal polysaccharide types 3, 6B, 7F, 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, 5, 20 and 20 µg/ml in PBS buffer for types 6B, 7F, 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 1:5000 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 monoclonal antibodies used were HASP4, PS7/19, PS14/4, PS19/5 and PS23/22 for types 6B, 7F, 14, 19F and 23F respectively.

For serotype 3, a similar ELISA was done except that immunoplates were first coated with methylated human serum albumin (1 µg/ml in PBS, 2 hours, 37°C) in order to improve the PS3 coating (2.5 µg/ml in PBS, overnight, 4°C). Monoclonal antibody PS3/6 was used as standard reference.

4. Cellular response

Spleen cells were isolated 14d Post II from groups immunized with FG containing formulations and from naïve mice for use as a negative control for the FG-specific cellular response analysis. Samples were analyzed for both FG-specific IFN-γ and IL-5 cytokines secretion.

Assays were performed as described in Example 1.

RESULTS

1. GROUPS.

19 groups (A to S) received two immunizations of various formulations containing either the 11-valent conjugated vaccine or FG or a combination of both antigens formulated with CpG or CpG alum with a dose range of CpG: 50, 10 or 2 µg. Groups A to R represent these combinations. Group S, FG Alum constitutes a control for anti-RSV analysis. Finally group T constitutes the negative control for the CMI analysis.

TABLE 4

Groups	Antigen	Adjuvant
A	SPN Undeca (0.1 µg)	CpG 50µg
B	SPN Undeca (0.1 µg)	CpG 10µg
C	SPN Undeca (0.1 µg)	CpG 2µg
D	FG (2 µg)	CpG 50µg
E	FG (2µg)	CpG 10µg
F	FG (2µg)	CpG 2µg
G	SPN Undeca(0.1µg)+FG(2µg)	CpG 50µg
H	SPN Undeca(0.1µg)+FG(2µg)	CpG 10µg
I	SPN Undeca(0.1µg)+FG(2µg)	CpG 2µg
J	SPN Undeca (0.1 µg)	CpG 50µg /Alum 50µg
K	SPN Undeca (0.1 µg)	CpG 10µg /Alum 50µg
L	SPN Undeca (0.1 µg)	CpG 2µg /Alum 50µg
M	FG (2 µg)	CpG 50µg /Alum 50µg
N	FG (2µg)	CpG 10µg /Alum 50µg
O	FG (2µg)	CpG 2µg /Alum 50µg
P	SPN Undeca(0.1µg)+FG(2µg)	CpG 50µg /Alum 50µg
Q	SPN Undeca(0.1µg)+FG(2µg)	CpG 10µg /Alum 50µg
R	SPN Undeca(0.1µg)+FG(2µg)	CpG 2µg /Alum 50µg
S	FG (2µg)	Alum
T	none	none

2. HUMORAL RESPONSE.

2.1. Anti-FG antibodies

None of the FG-specific responses were affected by the mixture with the 11-valent pneumococcal polysaccharide conjugate. For practical purposes the FG-specific analysis below are thus only described for the FG formulations.

Analysis of the specific anti-FG Ig antibodies at 14d/15 Post II vaccination was performed. The results show the induction of FG–specific Ig Ab with all formulations (**Figure 1**). For the FG CpG formulations the FG-specific Ig titers decrease with decreasing doses of CpG (groups D-F). The FG-CpG Alum based formulations induce higher FG-specific Ig Ab responses than those observed for the FG CpG or FG Alum group. A synergistic effect can be observed upon formulating FG with CpG and Alum as compared to the FG formulated with either alone. The Ab levels induced by FG-CpG Alum formulations are not sensitive to the CpG doses tested.

Analysis of the induced anti-RSV/A neutralizing antibodies leads to the same conclusions as for the anti-FG Ig antibody response (**Figure 2**).

Analysis of individual anti-FG IgG1 and IgG2a isotype titers showed similar antibody response profiles to the ones observed for the anti-FG Ig and neutralizing antibody titres for the FG CpG formulations (**Figure 3**). For the FG CpG Alum based formulations the Ab profiles were also similar except that a slight decrease in IgG2a antibodies could be observed with decreasing doses of CpG. Here too a synergistic effect is observed upon formulating FG with CpG and Alum as compared to the FG formulated with either CpG or alum alone. Further analysis showed that the addition of the 11-valent conjugated SP vaccine to FG CpG Alum or FG CpG does not affect the IgG1 and IgG2a responses.

Analysis of the ratio of IgG1/IgG2a isotype responses indicates that the greater the CpG dose the greater the amount of both isotypes and in particular the IgG2a antibodies in any of the tested formulations. In comparison to the CpG formulations, FG Alum induces much lower levels of IgG2a antibodies and much higher levels of IgG1 antibodies.

2.2. Anti-Pneumococcal Polysaccharide IgG

For both adjuvant formulations, anti-PS IgG titers were at least similar in animals given the 11-valent PS conjugate together with FG, compared to the animals immunized with the 11-valent PS conjugate alone (**Figure 4**). This result demonstrated that combining pneumococcal PS conjugates and RSV FG antigen within a CpG or an Alum CpG formulation did not reduce or alter the anti-pneumococcal immune response. Antibody titers to serotypes 7F and 14 were even significantly improved in the presence of FG using the CpG adjuvant formulation (with 2 µg and 50 µg CpG doses).

Higher antibody responses were reached to serotypes 7F, 14 and 19F (and to a lesser extent to serotypes 3 and 6B), when administering the Alum CpG formulation rather

than CpG given alone at the same dose. Using the former formulation, 2 μg CpG was as good an adjuvant as 50 μg CpG, whereas a clear dose-response was observed in animals given the non-adsorbed CpG formulations.

3. CELLULAR RESPONSE.

None of the FG-specific responses were affected by the mixture with the 11-valent pneumococcal polysaccharide conjugate. For practical purposes the FG-specific analysis below are thus only described for the FG formulations. Due to experimental problems no analysis was obtained from group G.

The analysis of the production of IL-5 and IFN-γ (**Figure 5**) shows that FG CpG formulations induce IFN-γ and a weak amount of IL-5. FG CpG alum formulations however do induce much higher amounts of IFN-γ independently of the CpG dose while IL-5 production seems to increase with decreasing CpG doses. This is reflected in the INF-γ/IL-5 ratio by a dominant IFN-γ production, marker of a Th1 response, for both formulations. However, the FG CpG alum formulations induce much more of the Th1 marker than FG CpG formulations alone.

DISCUSSION AND CONCLUSIONS

The analysis of the induced anti-FG Ig specific and anti-RSV/A neutralizing antibody titers shows that the combination of FG CpG alum and FG CpG with the *S. Pneumoniae* 11-valent conjugated vaccine does not hamper the induction of the humoral and cellular responses.

Thus the combination of FG CpG alum or FG CpG with Strep 11-valent conjugated vaccine does not affect the response observed with FG CpG alum or FG CpG and therefore the characteristics of these formulations eg induction of high secondary neutralizing antibody responses and a Th1 response as measured by the presence of IgG2a antibodies and the induction IFN-γ are maintained.

For practical purposes the conclusions are thus described below in relation to FG formulations.

The analysis of the induced anti-FG Ig specific and anti-RSV/A neutralizing antibody titers shows a dose dependent induction of these antibodies for FG CpG formulations which is not observed for the FG CpG alum formulations. In addition, a clear synergistic effect is observed upon formulating FG with CpG alum as compared to FG formulated with either CPG or alum alone.

Based on the isotype analysis FG CpG-Alum, FG CpG and FG alum all induce a mixed Th profile as measured by the induction of both IgG2a and IgG1 antibodies. FG alum however induces mostly IgG1 antibodies, marker of a Th2 response while FG CpG and FG CpG-alum both induce more IgG2a, marker of a Th1 response than IgG1. For both CpG formulations, the greater the CpG dose, the greater the predominance of the Th1 marker.

Analysis of the induced specific cell mediated response suggests that, similarly to what was observed for the FG-specific Ig isotype responses, both FG CpG alum and FG CpG formulations induce IFN-γ, marker of a Th1 response with FG CpG alum inducing the highest amounts.

Results demonstrated that combining pneumococcal PS conjugates and RSV FG antigen within a CpG or an Alum CpG formulation did not alter the anti-pneumococcal immune response. Antibody titers to serotypes 7F and 14 were even significantly improved in the presence of FG using the CpG adjuvant formulation. For several serotypes, the Alum CpG formulations were clearly more potent than CpG given alone at the same dose. Using the former formulation, 2 µg CpG was as good an adjuvant as 50 µg CpG, whereas a clear dose-response was observed in animals given the non-adsorbed CpG formulations.

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, and inactivated RSV.
3. A vaccine as claimed in any of claims 1 or 2 wherein the derivative is an antigen essentially devoid of a transmembrane domain.
4. A vaccine as claimed in any of 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 in any of claims 1 to 4 additionally comprising an aluminium salt or a saponin adjuvant.
6. A vaccine as claimed in any of claims 1 to 5 wherein the oligonucleotide comprises two CpG dincucleotides.
7. A vaccine as claimed in any of claims 1 to 6 wherein the CpG oligonucleotide is between 15-45 nucleotides in length.
8. A vaccine as claimed in any of claims 1 to 7 wherein the CpG oligonucleotide comprises at least one phosphorothioate internucleotide bond.
9. A vaccine as claimed in any of claims 1 to 8 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 any of claims 1 to 9 in which a *Streptococcus pneumoniae* antigen is additionally present.
11. A vaccine formulation as claimed in claim 10, wherein the *Streptococcus pneumoniae* antigen is the capsular polysaccharide.

12. A vaccine formulation according to claim 11, wherein the polysaccharide is conjugated to a protein.

13. A vaccine formulation as claimed in any of claims 1 to 12 in which an Influenza virus antigen is additionally present.

14. A vaccine formulation as claimed in any of claims 1 to 13 in which a Group B Streptococcus antigen is additionally present.

15. A vaccine formulation as claimed in any of claims 1 to 14 in which a PIV-3 antigen is additionally present.

16. A method for the prevention or amelioration of RSV infection of a patient, comprising administering an effective amount of a vaccine of any of claims 1 to 15 to patients.

17. A vaccine as claimed in any of claims 1 to 15 for use as a medicament.

18. A method of producing a vaccine as claimed in any of claims 1 to 15 comprising admixing a RSV antigen and a CpG immunostimulatory oligonucleotide.

Fig. 1

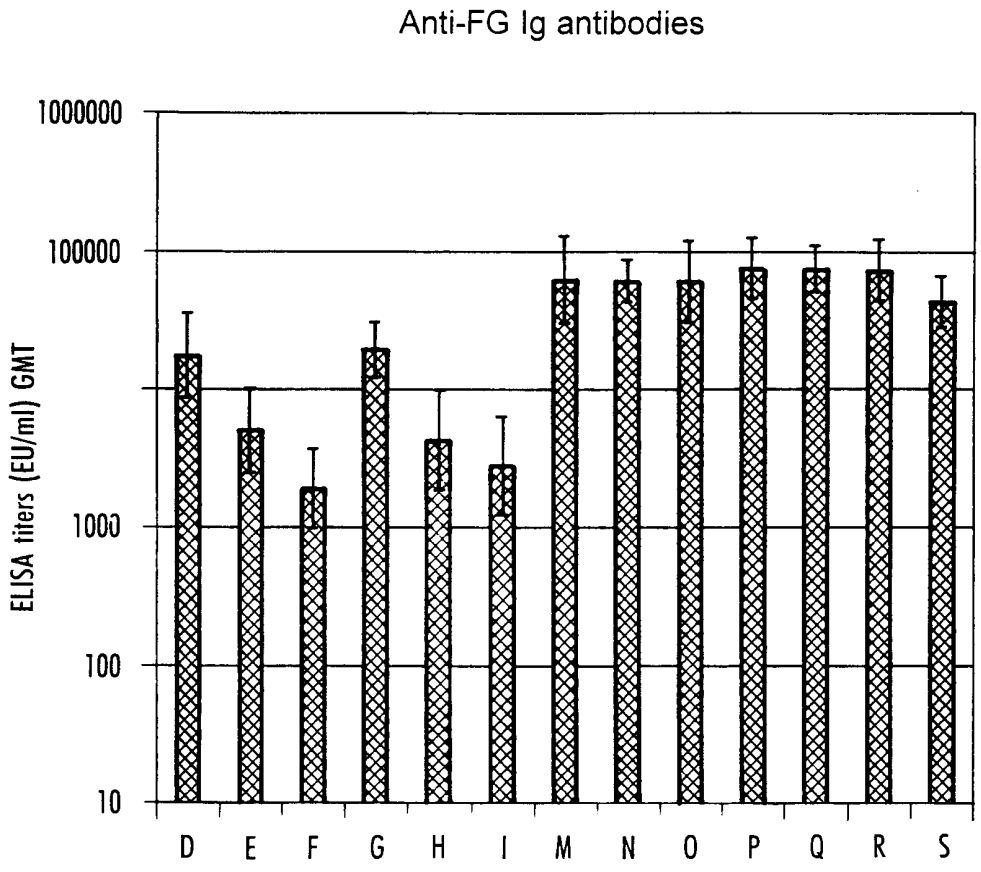
Anti-FG Ig antibodies
1000000
100000
1000
100
10
ELISA titers (EU/ml) GMT
D
E
F
G
H
I
M
N
O
P
Q
R
S

Fig. 2

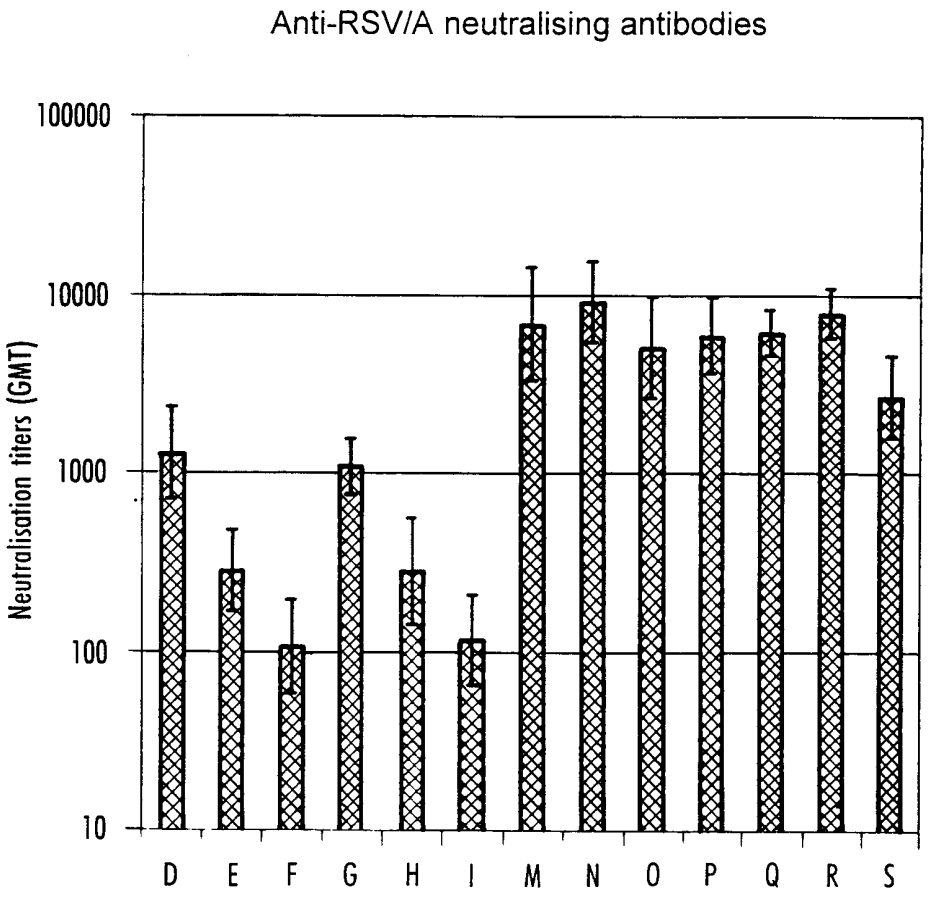
Anti-RSV/A neutralising antibodies
Neutralisation titers (GMT)
100000
10000
1000
100
10
D
E
F
G
H
I
M
N
O
P
Q
R
S

Fig. 3

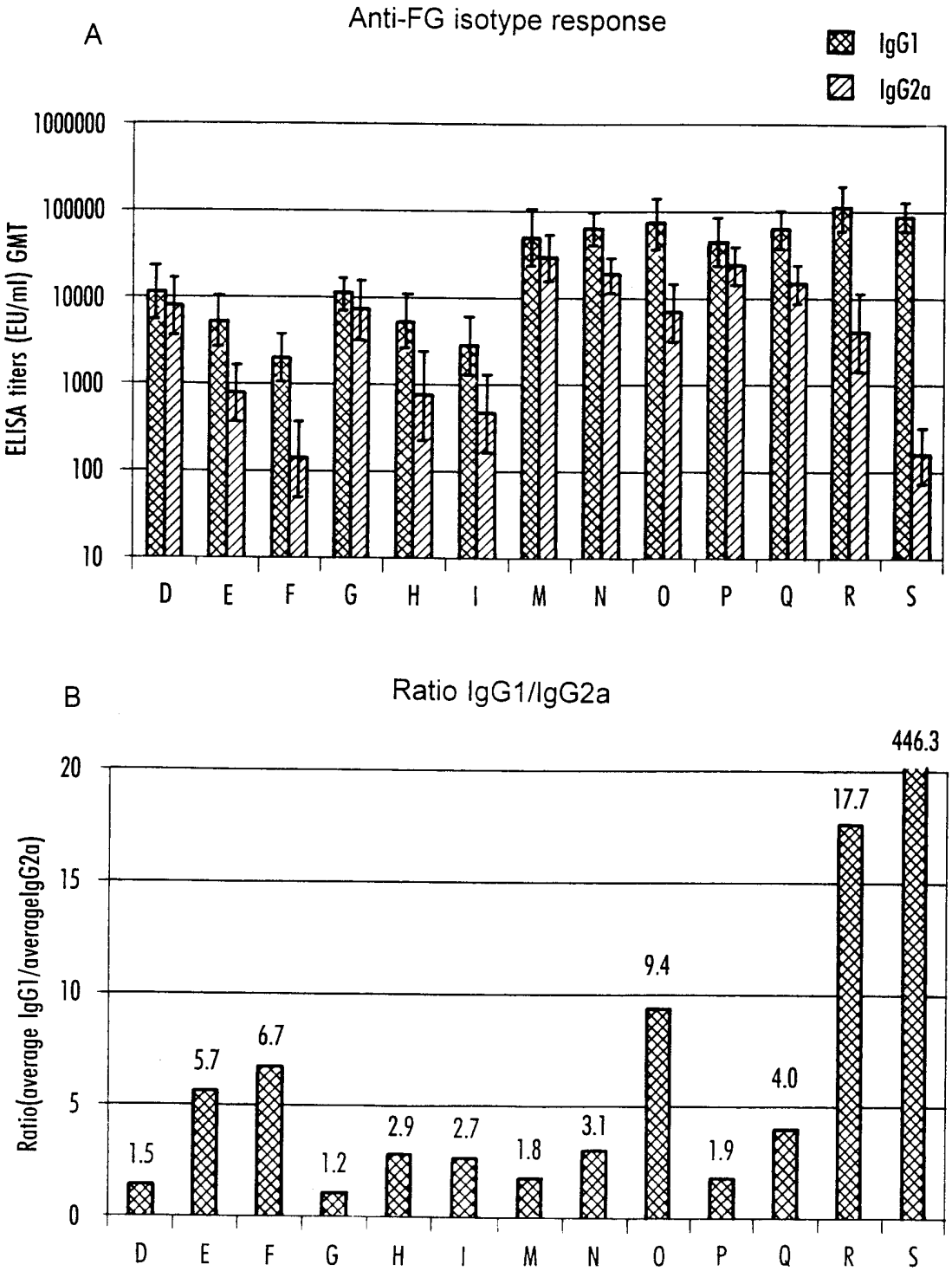
A
Anti-FG isotype response
IgG1
IgG2a
ELISA titers (EU/ml) GMT
1000000
100000
10000
1000
100
10
D E F G H I M N O P Q R S
B
Ratio IgG1/IgG2a
Ratio(average IgG1/averageIgG2a)
20
15
10
5
0
1.5
5.7
6.7
1.2
2.9
2.7
1.8
3.1
9.4
1.9
4.0
17.7
446.3
D E F G H I M N O P Q R S

Fig.4

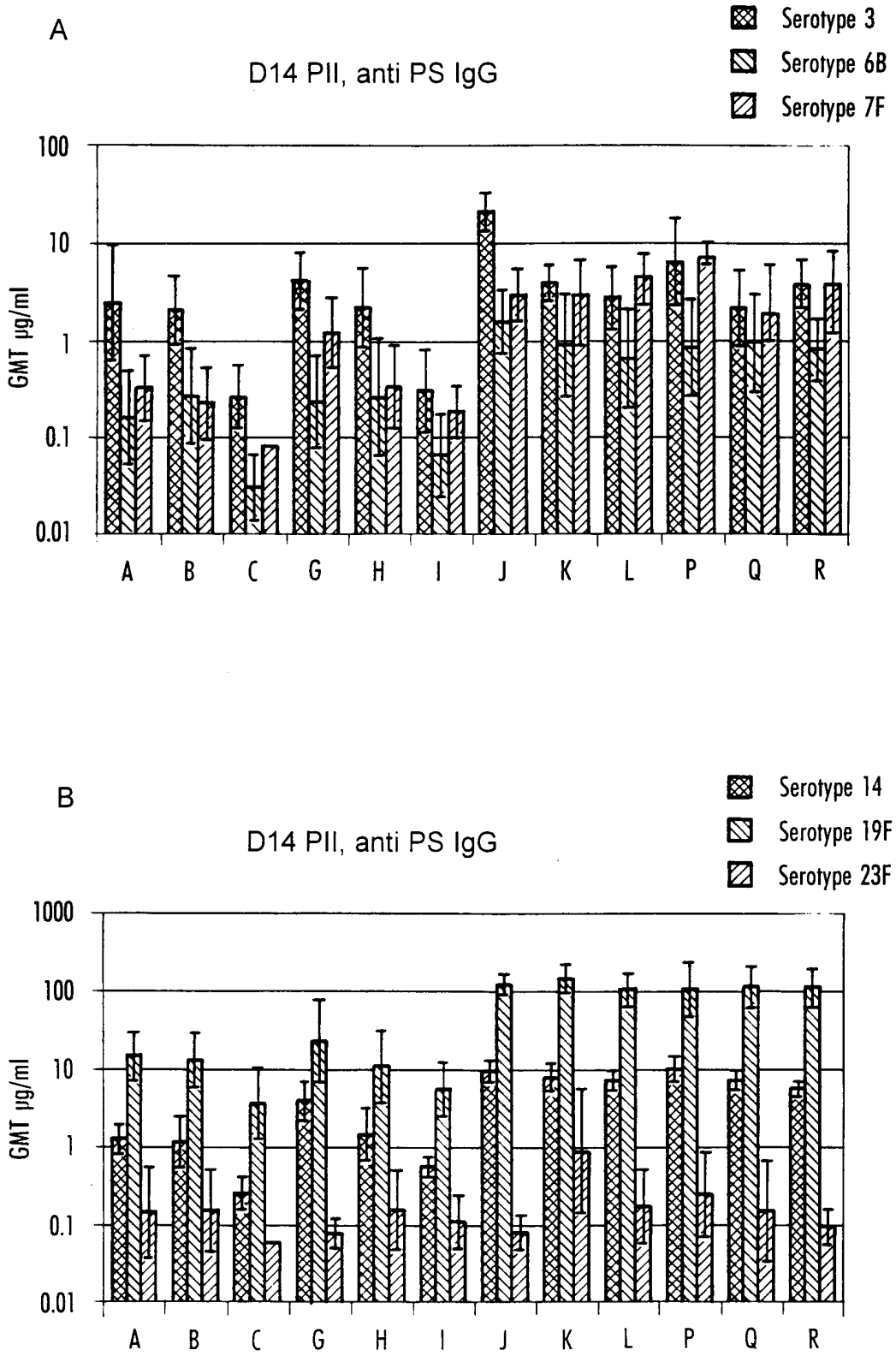
A
D14 PII, anti PS IgG
Serotype 3
Serotype 6B
Serotype 7F
GMT µg/ml
100
10
1
0.1
0.01
A B C G H I J K L P Q R
B
D14 PII, anti PS IgG
Serotype 14
Serotype 19F
Serotype 23F
GMT µg/ml
1000
100
10
1
0.1
0.01
A B C G H I J K L P Q R

Fig. 5 RSV/STREPNEU COMBINATION: CYTOKINES SECRETION ON SPLEEN CELLS