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(54) **MULTIVALENT LIPOSOMAL VACCINE COMPOSITIONS COMPRISING POLYSACCHARIDE ANTIGENS AND A PROTEIN ADJUVANT**

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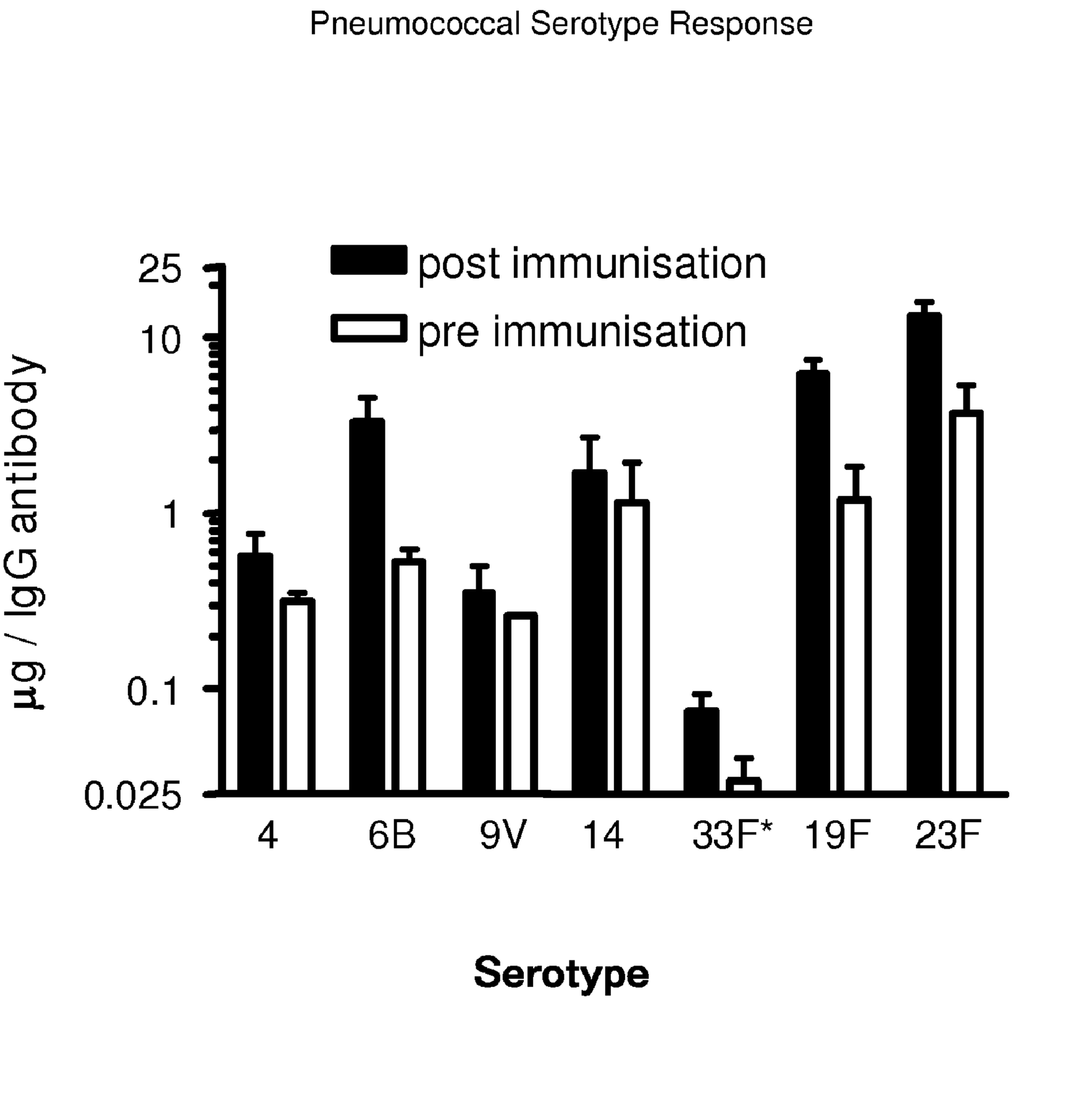
(57) **ABSTRACT**

A multivalent liposomal composition, preferably a vaccine, comprises liposomes formed of liposome forming compounds, containing coentrapped polysaccharide antigens of several serotypes and T-cell dependent protein carrier, such as tetanus toxoid. The invention is of use in the production of vaccines against *Haemophilus influenzae, Streptococcus pneumoniae* or *Neisseria meningitidis.*



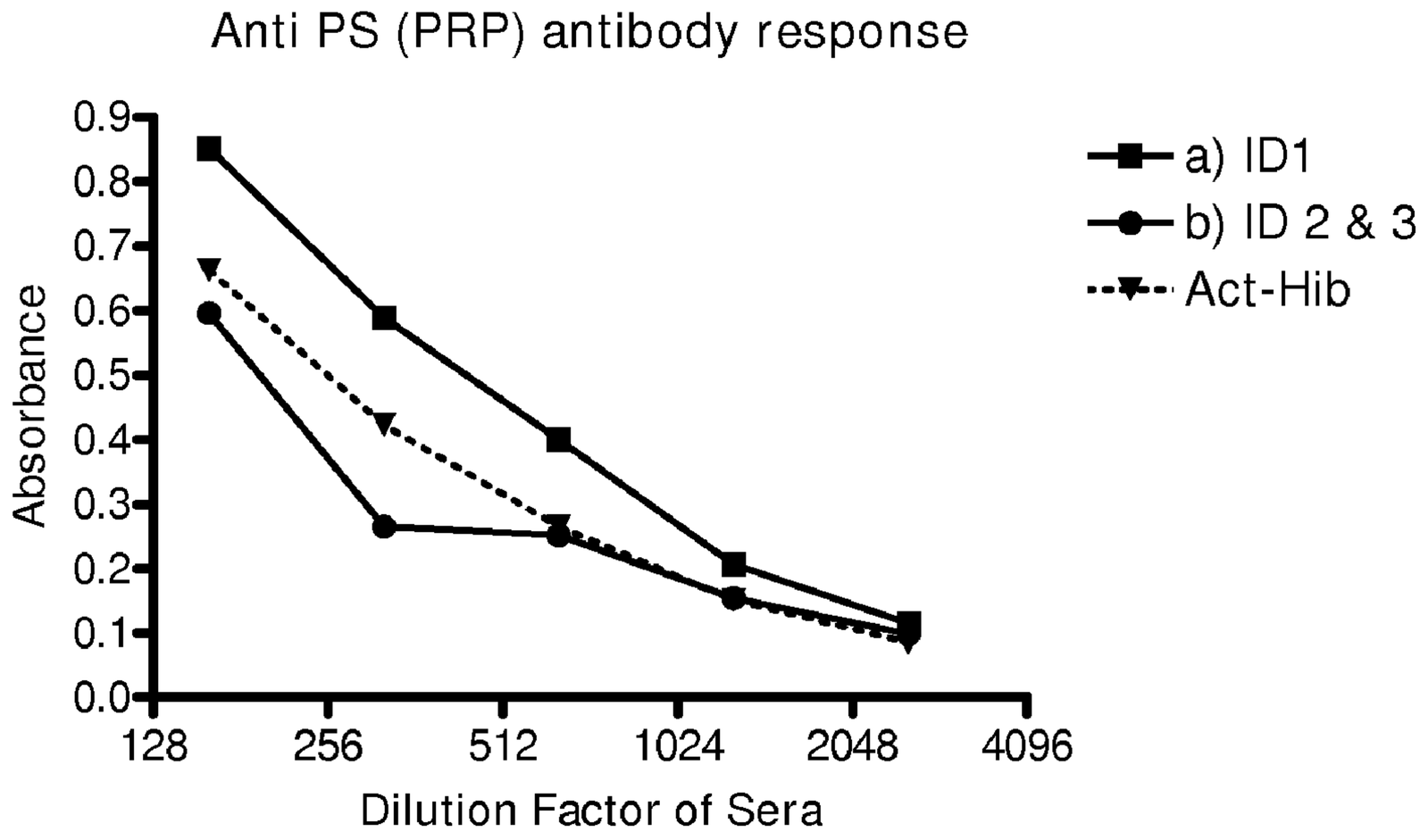


Figure 1

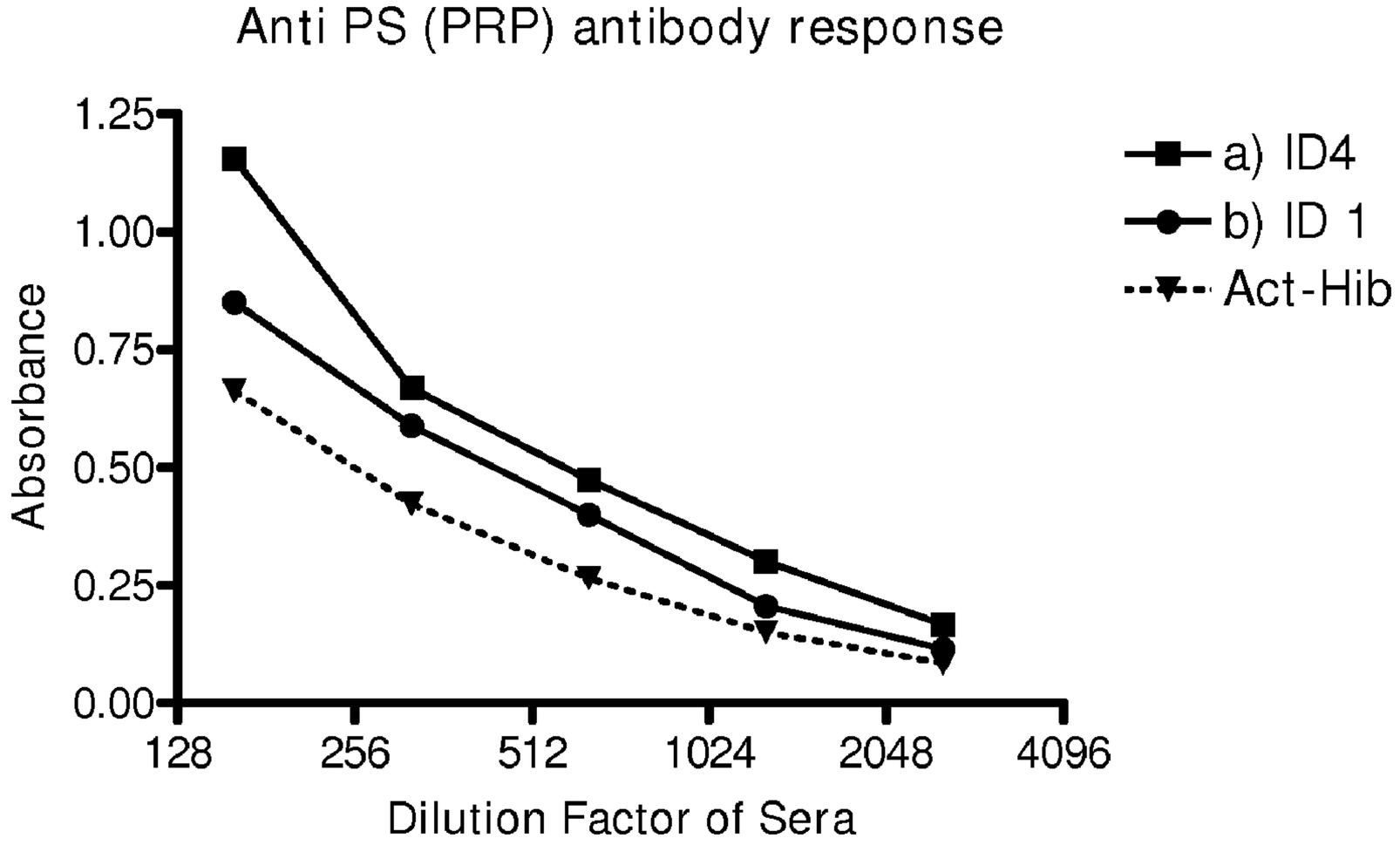


Figure 2

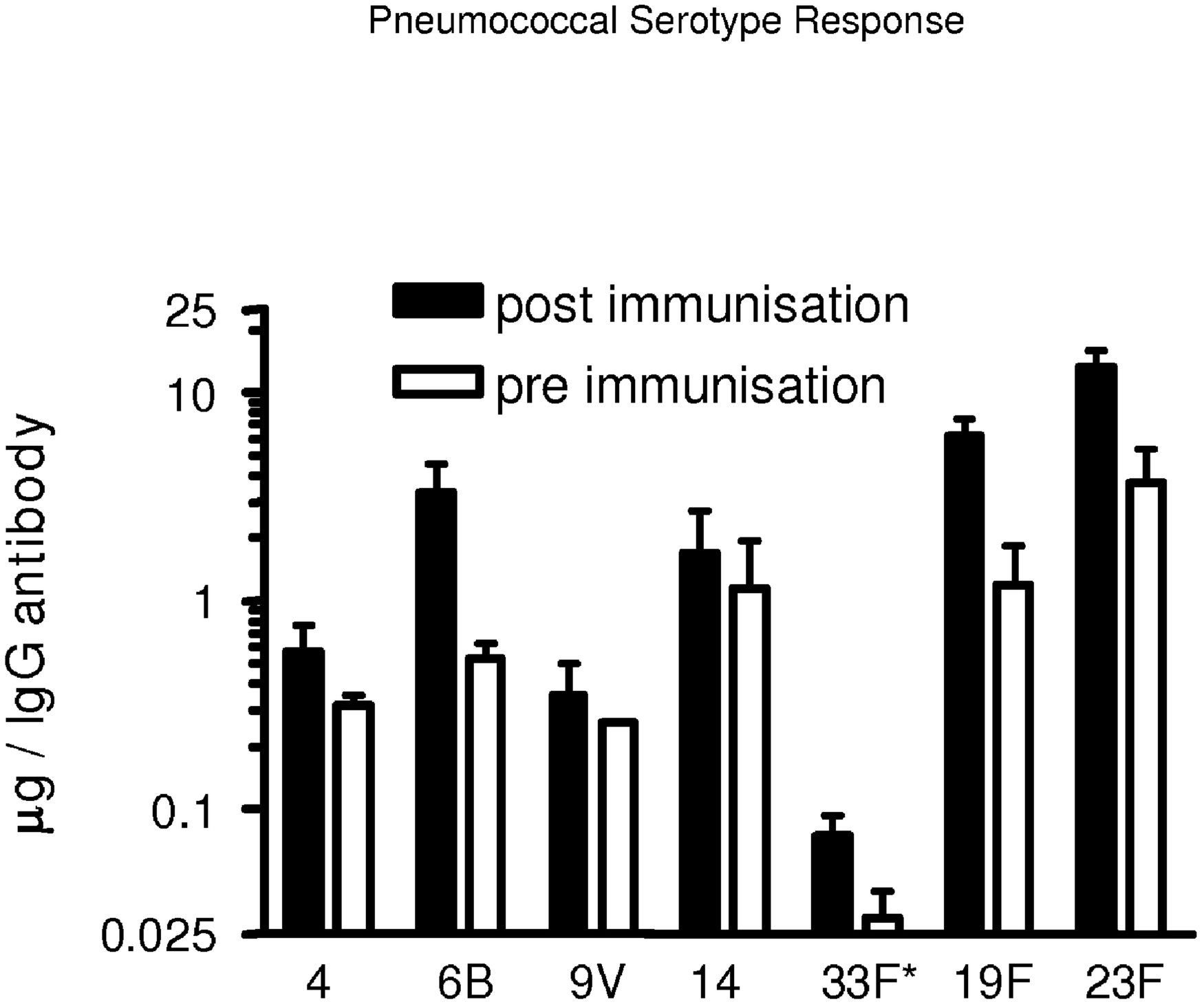

Pneumococcal Serotype Response
post immunisation
pre immunisation
µg / IgG antibody
25
10
1
0.1
0.025
4
6B
9V
14
33F*
19F
23F
Serotype

Figure 3

MULTIVALENT LIPOSOMAL VACCINE COMPOSITIONS COMPRISING POLYSACCHARIDE ANTIGENS AND A PROTEIN ADJUVANT

[0001] The present invention relates to liposomal compositions, in particular compositions useful for eliciting an immune response against polysaccharide antigens, in particular derived from pathogenic microbes, such as pneumococcal and Hib polysaccharide antigens.

[0002] Bacterial infections caused by encapsulated bacteria are a major world health problem. The species *Streptococcus pneumoniae, Haemophilus influenzae* and *Neisseria meningitidis* are difficult to vaccinate against due to the thymus independent nature of the major surface antigens, the capsular polysaccharides.

[0003] T-cell independent antigens present particular problems regarding the development of effective vaccines. Antibody production is low and is not normally boosted by re-immunisation. The antibody isotypes are restricted to IgM and other isotypes are generally of low affinity for a specific antigen.

[0004] A major problem lies in the response of young children to T-cell independent vaccines. These individuals are amongst the most vulnerable such bacterial infections. This age group responds most poorly to T-cell independent antigens.

[0005] Various methods have been attempted to adjuvantate polysaccharide antigens. For instance polysaccharides have been conjugated to carrier proteins such as tetanus toxoid, which results in some improvement in immunogenic effect. The polysaccharides have alternatively been formulated with liposomes, which also gives an enhanced immune response.

[0006] Burgeot, C. et al in Vaccine 2001, 19, 2092-2099, disclose immunopotentiation of polysaccharide vaccine from *Staphylococcus aureus* co-entrapped in liposomes with alpha toxin. The liposomes were formed of liposome forming compounds containing egg phosphatidylcholine, stearylamine and cholesterol in molar ratios of 7:2:1. The liposomes were formed using the dehydration rehydration method, with dehydration of a mixture of empty SUV and antigens suspended in a 10 mM Hepes (pH 7.4), 150 mM sodium chloride buffer. The ratio of polysaccharides to toxin protein ranged from 20 to 5. Alpha toxin is a highly toxic substance and is haemolytic. The authors reason that its activity in potentiating the antigenicity of the polysaccharide antigen is dependent upon the properties it exhibits as a toxin involving puncturing of the cell membrane. They show that the potentiation is not achieved with heat inactivated toxin derivative. Alpha toxin has a subunit molecular weight of about 33 kDa.

[0007] Pietrobon, P. J. F. et al in Immunomethods 4, 236-243 (1994) describe coentrapment into liposomes of the T independent antigen LPS (which is not water-soluble) and a polypeptide with T-cell recognition sites, haemagglutinin (HA). The liposome sizes are not disclosed. The weight ratio of LPS to HA is in the range 2:1 to 1:10. The liposomes are made by forming a lipid film containing LPS and HA and hydrating this in aqueous suspending liquid containing n-octylglucopyranoside. HA is not inert, but binds to various cell surface receptors via sialic acid bearing glycons and stimulates an innate immune response. Compounds which stimulate an innate immune response may generate undesirable inflammation.

[0008] According to the invention there is provided a new liposomal composition comprising two or more polysaccharide antigens and a non-toxic protein carrier having at least one T-cell epitope, wherein each polysaccharide antigen is coentrapped within liposomes with protein carrier and the liposomes are formed of liposome forming compounds comprising at least one phospholipid.

[0009] In the invention, the term "coentrapped" means that the two actives, namely the polysaccharide antigen and the protein carrier, must be associated with the same liposome. Although association may be solely with the outer surface of liposomes, it is preferred that the actives are at least in part entrapped in the intravesicular space of the liposomes. Both actives are preferably water-soluble and thus preferably located in the aqueous phase of the liposomal formulations. Where the liposomes are multi-lamellar the actives may be between lamellae.

[0010] In the invention, the composition is multivalent, that is it comprises several polysaccharide antigens in admixture. The vaccine comprises two or more, preferably three or more, five or more, seven or more, for instance up to forty, preferably in the range 7 to 23. Preferably, the antigens are derived from the same bacterial species. The antigens in such a multivalent composition may be co-encapsulated in liposomes, or there may be several populations, the liposomes of which each contain a separate antigen or antigen mixture distinct from the other populations. Preferably the antigens are separately entrapped in liposomes, that there is a district popoulation for each polysaccharide antigen. This allows optimum flexibility in terms of generating a variety of different thereof vaccines from common starting-materials. Conveniently the protein carrier is the same in each of several populations.

[0011] Although there may be benefit in conjugating (for example, via covalent attachment) the antigen to the adjuvant protein, preferably the invention avoids such conjugation. This has the great advantage of allowing coformulation of many ingredients especially different antigens to form a multivalent antigen composition.

[0012] The protein carrier is non-toxic to a mammalian body and should preferably not stimulate an innate immune response when administered thereto. Thus, the carrier is preferably effectively 'inert' in the mammalian body. Less preferably the protein acts as an adjuvant and is not biologically inert but engages (either directly, or via components that are released from killed host cells) host receptors to elicit cellular responses from the cells of the immune system in the form of an innate immune response.

[0013] The carrier protein has a molecular weight high enough for it to act as a T-cell dependent antigen. The molecular weight is preferably at least 35 kDa, for instance up to 1000 kDa, eg in the range 75-400 kDa. The present invention can use proteins which preferably have been used as carriers for T-cell independent antigens in the prior art, such as ovalbumin, tetanus toxoid and diphtheria toxoid. The protein should generally be non-haemolytic, and non-toxic . In terms of non-toxicity, for instance, the protein should have an LD50 in excess of 4 mg/kg (in mice, iv or sc). Preferably the protein should be level 6 on the Hodge & Sterner scale, ie be Relatively Harmless.

[0014] Suitable proteins include ovalbumin, tetanus toxoid, diphtheria toxoid and diphtheria CRM197 (a genetic mutant

of diphtheria toxin). Tetanus toxoid has a molecular weight of about 100 kDa. Tetanus toxoid and diptheria CRM197 (a genetic mutant of the toxin) fail to elicit the significant immune response elicited by the corresponding toxins. This is thought to be due to attenuation by formaldehyde or genetic mutation. These carriers do, however, elicit an adaptive immune response by activation and the subsequent proliferation of T-helper cells that are needed for antibody responses.

[0015] Preferably, the liposomal composition is in unit dosage form and comprises 1-30 μg of the protein carrier, more preferably 10-30 μg. Such dosages are believed to be suitable for administration to a subject in need of immunisation, such as a human.

[0016] In the invention, the polysaccharide antigens are all preferably derived from an infectious agent, preferably a pathogenic bacterium, for instance selected from *Haemophilus influenzae, Streptococcus pneumoniae, Neisseria meningitidis, E. coli,* or group B *Streptococcus*. Most preferably the antigens are derived from *Haemophilus influenzae* or *Streptococcus pneumoniae*. The polysaccharide antigens are preferably T-cell independent antigens. In the composition of the invention, the weight ratio of polysaccharide antigen to protein carrier is preferably in the range 6:1 to 1:6, more preferably 4:1 to 1:3. Preferably, there is an excess of polysaccharide to protein in the composition.

[0017] The liposomes preferably comprise compounds having no overall ionic charge. The compounds are preferably neutral, including zwitterionic compounds with one anionic and one cationic charge, but may contain small quantities of anionic or cationic compounds preferably being charge-balanced with oppositely charged compounds. Preferably the compounds comprise phosphatidylcholine compounds and/or phosphatidylethanolamine compounds. The liposome-forming compounds are usually amphiphilic, i.e. consist of a hydrophobic component and a hydrophilic component. The hydrophobic components are generally provided by acyl chains but the liposome-forming compounds may alternatively be glycerol-ether based lipid compounds. Non lipidic compounds, that is not based on glycerol compounds, may be included if desired, for instance non-ionic surfactant type materials. Preferably the compositions comprise cholesterol, for instance in a molar amount of at least 10%, preferably at least 25%, based on total liposome forming components.

[0018] Preferably the liposomes have an average diameter in the range 50-700 nm, more preferably 80-500 nm most preferably 100 to 300 nm. The average diameter is measured as disclosed in WO99/65465, by photon correlation spectroscopy.

[0019] The composition of the invention may be in the form of an aqueous suspension, that is in which the liposomes are suspended in a continuous aqueous medium. Alternatively the composition may be a precursor of such an aqueous composition, which may be diluted with water or an aqueous liquid to form the aqueous suspension. Such precursors may be in the form of dried materials, especially in powder form, for instance provided by spray drying or freeze drying (lyophilisation).

[0020] The compositions of the invention may be pharmaceutical compositions further comprising (a) pharmaceutically acceptable diluent(s) and/or excipient(s). Since the compositions are intended to be used directly or after intermediate formulation steps, to be administered to mammalian subjects, the diluents or the excipients are preferably pharmaceutically acceptable. Suitable excipients are known.

[0021] It is particularly preferred that the composition comprises sugars. Sugars may assist stabilisation of the liposomes during formation of the liposomes and/or in storage. Preferably the liposomes are formed by the dehydration-rehydration method. Empty small unilamellar vesicles (SUV's) (e.g. made by hydrating a lipid film to form multi-lamellar vesicles and sonicating to form SUVs) and active (polysaccharide antigen and protein carrier) are suspended in an aqueous liquid prior to drying preferably by lyophilisation. The dried product is then rehydrated and optionally subjected to steps to remove non-entrapped material, over-sized liposomes or to reduce the average liposome size, by methods known to the person skilled in the art.

[0022] For improved control of rehydrated vesicle size, the method used includes sugar in the suspending liquid of the dehydration step, as discussed in WO99/65465. The sugar may be selected from monosaccharides such as glucose and fructose, disaccharides such as lactose and sucrose as well as polysaccharides. A particularly preferred sugar is a di-saccharide such as trehalose, sucrose or lactose or a monosaccharide such as glucose. In particular the preferred sugar is sucrose.

[0023] In such methods, the amount of sugar is such that mass ratio of sugar to liposome forming compound is in the range of from 1:1 to 6:1 w/w, most preferably from 1:1 to 5:1. With higher levels of sugar, the encapsulation efficiency of the method is reduced. With lower amounts of sugar, however, the control over the size of the final liposomes is lost.

[0024] The invention further provides a process for process for producing the new composition in which the polysaccaride antigen and protein carrier are co-encapsulated in liposomes.

[0025] Preferably the methods described above are used for the encapsulation. Sugar need not be used but preferably is used in these dehydration-rehydration methods. The empty liposomes are preferably small or medium sized and may be multilamellar or unilamellar.

[0026] The product liposomes preferably have average diameter in the range 50 to 700 nm, more preferably in the range 80 to 500 nm, most preferably in the range 80 to 300 nm. Preferably the composition contains very low levels of liposomes with diameters greater than 1500 nm, even more preferably very low levels of liposomes with diameters greater than 1000 nm. The level of very small liposomes, e.g. less than 20 nm should also be kept as low as possible.

[0027] The invention also provides the use of the liposomes and compositions in the manufacture of a medicament for administration to a mammal to elicit an immune response to the polysaccharide antigen.

[0028] Preferably the immune response involves production of IgG at least, to the polysaccharide antigen, and additionally preferably IgM and IgA, preferably so that a protective effect to a challenge from the infectious micro organism is achieved in the recipient.

[0029] Preferably the composition is administered subcutaneously, intravenously, intraperitoneally, intramuscularly, intranasally, by inhalation, intravaginally, buccally or orally. Most preferably the composition is administered subcutaneously.

[0030] Generally the administration is for protective purposes to provide a response against infection by an infectious microbe, especially an infectious bacterium, of the type men-

tioned above. The subject to which the composition is administered may be a mammal of any age, in need of having protective immunity. The invention is of most value for treating humans. For instance administration may be to provide resistance to seasonal outbreaks, specific outbreaks of the infections, or may be part of health programmes especially for infants. The invention is of particular value for the treatment of human infants having reduced immune responses to T-cell independent antigens, for instance polysaccharide antigens, for instance being less than two years of age.

[0031] The invention further provides methods of administering the compositions.

[0032] Further preferred embodiments of the invention are mentioned in the claims.

[0033] The following examples illustrates the invention and refer to the following figures, in which

[0034] FIG. **1** shows the Anti PS (PRP) antibody response of mice when administered formulations ID1, ID2 & 3 and Act-Hib without cholesterol (Example 1);

[0035] FIG. **2** shows the Anti PS (PRP) antibody response of mice when adminstered formulations ID4, ID1, and Act-Hib with cholesterol (Example 1); and

[0036] FIG. **3** shows the IgG antibody response pre and post immunisation to seven serotypes tested (Example 2).

Example 1

Reference

[0037] Demonstration of anti polysaccharide response, in mice, to liposomal polysaccharide vaccines in compared to a licensed (in man) polysaccharide vaccine delivery of polysaccharide.

Experimental Details:

[0038] The following liposome formulations (ID1-6) were prepared, Table 1, using a conventional lipid film dry/hydration to MLV/sonication to small unilamellar vesicles (SUV)/DRV process (WO9965465). Sucrose x3 lipid mass was added to the SUV and material(s) to be entrapped at the SUV stage prior to DRV process to reduce the size of the resultant liposomes formed post the DRV stage. The average diameter of the DRV by photon correlation spectroscopy is about 500 nm.

TABLE 1

Liposomal formulations prepared. (ID1 -6)

	Materials entrapped ([a] = co-entrapped)		
Lipid composition (molar ratio)	Tetanus Toxoid and PS (Hib PRP) [a]	Tetanus Toxoid	PS (Hib PRP)
EPC:DOPE:DOTAP (4:2:1)	ID1	ID2	ID3
Soya PC:Cholesterol (11:9)	ID4	ND	ND

Material Details:

[0039] The lipidic materials Egg PC (EPC, 98% purity, Lipoid) and Hydrogenated Soya PC (SPC-3.98% purity, Lipoid) were obtained form Lipoid GmbH. Whilst dioleoyloxyphosphatidylethanolamine (DOPE) (Avanti Product Number: 850725) were obtained form Avanti® Polar Lipids and, 1,2-dioleoyloxy-3-trimethylammonium propane (DOTAP) was obtained form Merck Chemicals Ltd. Purified polyribose ribitol phosphate capsular polysaccharide (PRP-PS) of *Haemophilus influenzae* type b and Tetanus Toxoid material were obtained from the Serum Institute of India (SII). The entrapment efficacies were also tested. The efficacy for both PS and protein for ID1 was around 90% or higher. The efficacy for ID4 for both materials was around 70%.

[0040] The EPC without cholesterol formulations in Table 1 were tested for their ability to induce a antibody response to the polysaccharide antigen in mice. Mice (CD-1), were dosed once subcutaneously with a) formulation ID1 or b) formulations ID2 and 3, mixed immediately prior to dosing. A licensed vaccine product for the induction of protective levels of anti PRP (PS) antibodies in humans Act-Hib® (a vaccine of purified polyribose ribitol phosphate capsular polysaccharide (PRP) of *Haemophilus influenzae* type b, covalently bound to tetanus toxoid protein) was used as a comparator positive control. All doses were normalised with respect to the dose of PRP (PS) and TT administered, 2 and 4 μg respectively.

[0041] The results are shown in FIG. **1**.

Comments

[0042] The results of the anti PS (PRP) antibodies generated following immunisation with a) ID1, b) ID2 & 3 and , Act-Hib® clearly indicate that:

[0043] a) the co-delivered TT and PS (PRP) with the same liposomal vehicle (ID1) formulation produce a higher antibody response than the same materials TT and PS (PRP) dose but administered in separate liposomal vehicles (ID2 and 3 respectively); and

[0044] b) the co-delivered TT and PS (PRP) with the same liposomal vehicle (ID1) formulation produce a higher antibody response than the same materials TT and PS (PRP) dose administered in the form of a protein polysaccharide conjugate vaccine (Act-Hib®).

[0045] The Soya PC with Cholesterol formulations in Table 1 were tested for their ability to induce a antibody response to the polysaccharide antigen in mice. Mice (CD-1), were dosed once subcutaneously with a) formulation ID4 or b) formulations ID1, mixed immediately prior to dosing. A licensed vaccine product for the induction of protective levels of anti PRP (PS) antibodies in humans Act-Hib® (a vaccine of purified polyribose ribitol phosphate capsular polysaccharide (PRP) of *Haemophilus influenzae* type b, covalently bound to tetanus (toxiod) protein) was used as a comparator positive control. All doses were normalised with respect to the dose of PRP (PS) and TT administered, 2 and 4 m respectively.

[0046] The results are shown in FIG. **2**.

Comments

[0047] The results of the anti PS (PRP) antibodies generated following immunisation with a) ID4, b) ID1 and , Act-Hib® clearly indicate that:

[0048] a) the co-delivered TT and PS (PRP) with the same liposomal vehicle (ID3) formulation (with Cholesterol) produce a higher antibody response than the same materials TT and PS (PRP) dose administered in the form of a protein polysaccharide conjugate vaccine (Act-Hib®); and

[0049] b) the co-delivered TT and PS (PRP) with the same liposomal vehicle (ID4) formulation (with Choles-

terol) produce a higher antibody response than the same materials TT and PS (PRP) dose administered in a liposomal formulation (ID1) without cholesterol.

EXAMPLE 2

[0050] Demonstration of multivalent anti *Streptococcus pneumoniae* polysaccharide serum IgG reponses, in mice, following immunisation of a blend (multivalent) of monoserotype liposomal compositions comprising of coentrapped pneumococcal polysaccharide and protein diphtheria toxin (197) CRM Mutant within liposomes.

Experimental Details:

[0051] The following liposome formulations (ID7-20) were prepared, Table 2, using a conventional lipid film dry/hydration to MLV/sonication to SUV/DRV process (i.e. as in Example 1). Sucrose x3 lipid mass (SUV) was added to the SUV and material(s) to be entrapped at the SUV stage prior to DRV process to reduce the size of the resultant liposomes formed post the DRV stage.

[0052] Each formulation (ID7-20), consisted of an individual pneumococcal polysaccharide serotype coentrapped with the protein diphtheria toxin CRM197 mutant within liposomes. The mutant form of diphtheria toxin is described and was isolated as Uchida, Jr., T., Pappenheimer, Jr., A. M., Greany, R., (1973) J. Biol. Chem. 248, 3838-3844. and Uchida, Jr., T., Pappenheimer, Jr., A. M., Harper, A. A., (1973) J. Biol. Chem. B 248, 3845-3850. CRM197 is a non-toxic DT mutant containing a lesion in the A chain blocking ADP-ribosylation. CRM results from a base change in the structural gene resulting in the substitution of glutamic acid for glycine. While CRM shows no enzymatic activity, it is immunologically identical to diphtheria toxin. (Pappenheimer, Jr., A. M., Uchida, T. and Harper, A. A. (1972) Immunochem. 9, 891-906.)

[0053] CRM197 is similar to diphtheria toxoid. CRM197 is a well defined protein in contrast to formaldehyde treated toxin (toxoid) which is non-specifically cross linked. On SDS gels, the CRM197 protein migrates as a single major band of approximate molecular weight 63,000 daltons

TABLE 2

Liposomal mono PS entrapped formulations prepared. (ID7-20)

Formulation ID	Lipid (SUV), mg	Diphtheria Toxin CRM197, µg	Pneumococcal Polysaccharide µg	(Serotype)
ID7	7.81	21	84	1
ID8	7.81	21	84	2
ID9	7.81	21	84	4
ID10	7.81	21	84	5
ID11	7.81	21	84	6B
ID12	7.81	21	84	7F
ID13	7.81	21	84	9N
ID14	7.81	21	84	9V
ID15	7.81	21	84	12F
ID16	7.81	21	84	14
ID17	7.81	21	84	15B
ID18	7.81	21	84	19F
ID19	7.81	21	84	23F
ID20	7.81	21	84	33F

Material Details:

[0054] The lipidic materials used were Egg PC (E PC, 0.98% purity, Lipiod) and cholesterol (Sigma). Pneumococcal capsular polysaccharide (Pn PS) equivalent to the American Type Culture Collection (ATCC).materials and diphtheria toxin CRM197 protein were obtained from the Serum Institute of India Limited (SIIL).

[0055] The liposomal mono PS entrapped formulations (ID7-20) were individually rehydrated (DRV process) and pooled together to make a multivalent formulation immediately before immunization, via subcutaneous injection, of five CD1 mice (female). The dose of the multivalent vaccine contained the equivalent of 14 monovalent formulations containing 6 µg pneumococcal polysaccharide and 1.5 µg diphtheria toxin CRM197 protein and 0.559 mg of lipid. Consequently, the multivalent (14 serotypes) vaccine administered to the mice contain in total 84 µg pneumococcal polysaccharide and 21 µg diphtheria toxin CRM197 protein and 7.81 mg of lipid.

[0056] The mice received a total of two immunisation doses 21 days apart, additionally sera samples via superficial tail vein sampling were taken from the mice prior to the first immunisation dose and 14 days after the second dose.

[0057] The mouse sera samples obtained were assayed for some of the *Streptococcus pneumoniae* antibodies, IgG (7 Serotypes: 4, 6B, 9V, 14, 18C (detects 33F responses), 19F and 23F), by multi-analyte fluorescent detection (Luminex). This work was performed at the National Institute for Biological Standards and Control) (NIBSC). For the other serotypes no kit was available and so the responses were not measured.

[0058] The Luminex multi-analyte fluorescent detection is based on microscopic beads (microspheres) internally labelled with two different fluorophores. When excited by a 635 nm laser, the fluorophores emit at different wavelengths, 658 nm and 712 nm. By varying the 658/712 emission ratios, an array of up to 100 different fluorescent profiles, called classifications, can be created. Using precision fluidics, digital signal processors, and advanced optics, the Luminex 100 analyser assigns each microsphere according to its predefined classification. Thus, multiple-bead classifications can be combined in a single sample. In the assay employed each individual pneumococcal polysaccharide serotype is covalently attached to a microsphere with a unique classification. During the assay procedure, all the different pneumococcal polysaccharide serotype microspheres are combined with a single dilution of test murine sample. Antibodies in the murine sample attach to pneumococcal polysaccharide antigens on the microspheres. The amount of antibody bound to the microspheres is determined with anti-mouse IgG conjugated to phycoerythrin. When excited at 532 nm, phycoerythrin emits at 575 nm. The fluorescence intensity at 575 nm is directly proportional to the amount of antibody bound to the microspheres. Since the analyte specificity and position of each bead classification in the array is known, a single fluorescent reporter molecule can be used to calculate specific antibody levels to all serotypes tested (represented by the polysaccharide-bead conjugates).

Results:

[0059] FIG. **3** shows the calculated *streptococcus pneumoniae* antibodies, murine IgG (Serotypes specific responses: 4, 6B, 9V, 14, 18C (*see FIG. **3**, detects 33F responses), 19F and

23F), by multi-analyte fluorescent detection. Bars represent the average response of the 5 mice dosed and the error bars are standard deviations, the empty bars are pre immunisation background response of the mice whilst the solid bar is the response following the immunisation schedule.

Comments

[0060] The average response to all the seven serotypes tested were raised following immunisation with the blend (multivalent) of monoserotype liposomal compositions comprising of coentrapped pneumococcal polysaccharide and protein diphtheria toxin (197) CRM mutant within liposomes. The increase in response, post immunisation, relative to the pre immunisation response, was 1.8, 6.3, 1.4, 1.5, 2.5, 5.3 and 3.6 fold for the serotypes 4, 6B, 9V, 14, 33F, 19F and 23F fold respectively.

[0061] Thus the composition described demonstrates the ability to raise an antibody response to multivalent pneumococcal polysaccharide antigens in mice following immunisation with a blend (multivalent) of monoserotype liposomal compositions comprising coentrapped pneumococcal polysaccharide and protein diphtheria toxin (197) CRM mutant within liposomes.

Example 3

[0062] Demonstration of Protective Efficacy to live *Streptococcus pneumoniae* (challenge) in mice, following immunisation of a blend (multivalent) of monoserotype liposomal compositions comprising coentrapped pneumococcal polysaccharide and protein Diphtheria Toxin (197) CRM Mutant within the liposomes with a pneumococcal polysaccharide vaccine (Pneumovax) boost.

Experimental Details:

[0063] The following liposome formulations (ID21-34) were prepared, Table 3, using a conventional lipid film dry/hydration to MLV/sonication to SUV/DRV process (as in Example 1). Sucrose x3 lipid mass (SUV) was added to the SUV and material(s) to be entrapped at the SUV stage prior to DRV process to reduce the size of the resultant liposomes formed post the DRV stage.

[0064] Each formulation (ID21-34), consisted of an individual pneumococcal polysaccharide serotype coentrapped with a protein Diphtheria Toxin CRM197 mutant within liposomes.

TABLE 3

Liposomal mono PS entrapped formulations prepared. (ID21-34)

	Lipid (SUV),	Diphtheria Toxin	Pneumococcal Polysaccharide	
Formulation ID	mg	CRM197, μg	μg	(Serotype)
ID21	16.6	158.4	66	1
ID22	16.6	158.4	66	2
ID23	16.6	158.4	66	4
ID24	16.6	158.4	66	5
ID25	16.6	158.4	66	6B
ID26	16.6	158.4	66	7F
ID27	16.6	158.4	66	9N
ID28	16.6	158.4	66	9V
ID29	16.6	158.4	66	12F
ID30	16.6	158.4	66	14
ID31	16.6	158.4	66	15B

TABLE 3-continued

Liposomal mono PS entrapped formulations prepared. (ID21-34)

	Lipid (SUV),	Diphtheria Toxin	Pneumococcal Polysaccharide	
Formulation ID	mg	CRM197, μg	μg	(Serotype)
ID32	16.6	158.4	66	19F
ID33	16.6	158.4	66	23F
ID34	16.6	158.4	66	33F

Material Details:

[0065] The lipidic materials used were Egg PC (E PC, 98% purity, Lipoid) and Cholesterol (Sigma). Pneumococcal capsular polysaccharide (Pn PS) equivalent to the American Type Culture Collection (ATCC) materials and Diphtheria Toxin CRM197 protein were obtained from the Serum Institute of India Limited (SIIL).

[0066] The liposomal mono PS entrapped formulations (ID21-34) were individually rehydrated (DRV process) and pooled together to make a multivalent formulation immediately for immunisation, via subcutaneous injection, of ten Balb/C mice (female). The dose of multivalent vaccine contained the equivalent of 14 monovalent formulations containing 0.2 μg Pneumococcal Polysaccharide and 0.48 μg Diphtheria Toxin CRM197 protein and 0.138 mg of lipid. Consequently, the multivalent (14 serotypes) vaccine administered to the mice contain in total 2.8 μg Pneumococcal Polysaccharide and 6.72 μg Diphtheria Toxin CRM1 97 protein and 1.935 mg of lipid.

[0067] Two groups of mice were immunised:

[0068] Group 1 were immunised, 14 day interval between doses, with 3 doses of the blend (multivalent) of monoserotype liposomal compositions, followed by pneumococcal polysaccharide vaccine (Pneumovax) boost administered 8 weeks after the last liposomal formulation dose.

[0069] The Pneumovax dose administered consists of a mixture of highly purified capsular polysaccharides from 23 pneumococcal types of *Streptococcus pneumoniae,* serotypes (1 2 3 4 5 6B 7F 8 9N 9V 10A 11A12F 14 15B 17F 18C 19F 19A20 22F23 F & 33F). The dose level administered for each purified capsular polysaccharides serotype was 7.5 μg and consequently the total polysaccharide dose administered was Pneumovax was 172.5 μg.

[0070] Group 2 were immunised, 14 day interval between doses, with 3 doses of pneumococcal polysaccharide vaccine (Pneumovax), followed by pneumococcal polysaccharide vaccine (Pneumovax) boost administered 8 weeks after the third Pneumovax dose.

[0071] Live *Streptococcus pneumoniae* challenge, was performed on all mice four weeks following the last dose. Briefly, mice were challenged intraperitoneally with 0.5 ml *S. pneumoniae* (serotype 6B) suspension prepared from fresh overnight colonies from a 5% horse blood agar plate were suspended in beef broth and further diluted to 4.0×10^7 CFU/ml.

[0072] The mice were observed for 6 days following administration of the bacterial challenge, and scored 0-4 based on their behaviour and clinical signs as below.

[0073] Score 0: healthy

[0074] Score 1: minor clinical signs of infection and inflammation e.g. observations of minor sign of distress and pain, changed activity, and social withdrawal.

[0075] Score 2: sever signs of infection like stiff movements, lack of curiosity, forced ventilation, changed body position, piloerection in the skin, or changes in movement.

[0076] Score 3: sever pain and the mouse was sacrificed immediately to minimise the suffering of the animal.

[0077] Score 4: the mouse was dead.

Results:

[0078] The results are shown in Tables 4 and 5, of observed responses in mice following live *Streptococcus pneumoniae* challenge.

TABLE 4

	Group 1									
	Day	Day		Day		Day	Day	Day	Day	
mouse	0	1		2		3	4	5	6	
no.	pm	am	pm	am	pm	am	am	am	am	Survival
1	1	1	2	1	1	1	1	0	0	56%
2	1	1	1	0	0	0	0	0	0	
3	1	4								
4	1	4								
5	1	4								
6	0	1	2	1	1	2	1	1	1	
7	Died before challenge									
8	0	1	2	0	0	0	0	0	0	
9	0	0	0	0	0	0	0	0	0	
10	0	2	3							

TABLE 5

	Group 2									
	Day	Day		Day		Day	Day	Day	Day	
mouse	0	1		2		3	4	5	6	
no.	pm	am	pm	am	pm	am	am	am	am	Survival
1	0	0	0	1	2	3				11%
2	0	0	0	0	0	1	1	1	1	
3	1	3								
4	0	3								
5	Died before challenge									
6	2	3								
7	2	4								
8	1	1	1	4						
9	2	4								
10	2	4								

Comments:

[0079] The results demonstrate that mice are better protected against live *Streptococcus pneumoniae* challenge (serotype 6B), following immunisation of a blend (multivalent) of monoserotype liposomal compositions comprising of coentrapped pneumococcal polysaccharide and protein Diphtheria Toxin (197) CRM Mutant within liposomes with a pneumococcal polysaccharide vaccine (Pneumovax) boost compared to immunisation with pneumococcal polysaccharide vaccine (Pneumovax) alone.

1. A multivalent liposomal composition comprising two or more polysaccharide antigens and a non-toxic protein carrier having at least one T-cell epitope, wherein each polysaccharide antigen is coentrapped within liposomes with protein carrier and the liposomes are formed of liposome forming compounds comprising at least one phospholipid.

2. The composition of claim **1**, wherein each antigen is encapsulated in a separate population of liposomes.

3. The composition of claim **1**, wherein several antigens are co-encapsulated in the same liposomes of the population.

4. The composition of claim **1**, comprising 7-23 polysaccharide antigens.

5. The composition of claim **1**, wherein the polysaccharide antigen is a T-cell independent antigen.

6. The composition of claim **5**, wherein the polysaccharide antigen is derived from an infectious agent.

7. The composition of claim **1**, wherein the antigens are derived from the same bacterial species.

8. The composition of claim **1**, wherein the polysaccharide antigen has no overall ionic charge.

9. The composition of claim **1**, wherein the protein is not covalently linked to the antigen.

10. The composition of claim **1**, wherein the protein carrier has a molecular weight of at least 35 kDa.

11. The composition of claim **1**, wherein the protein is selected from ovalbumin, tetanus toxoid and diphtheria toxoid, preferably tetanus toxoid.

12. The composition of claim **1**, wherein the ratio of polysaccharide antigen to protein carrier is 6:1 to 1:6.

13. The composition of claim **12**, wherein the ratio of polysaccharide antigen to protein carrier is in the range 4:1 to 1:3.

14. The composition of claim **1**, in unit dosage form, comprising 1-30 μg protein carrier.

15. The composition of claim **1**, wherein the liposome forming compounds comprise cholesterol.

16. The composition of claim **1**, wherein the liposome forming compounds in combination have no overall charge.

17. The composition of claim **1**, wherein the liposome forming compounds include at least one cationically charged compound.

18. The composition of claim **1**, wherein the phospholipids include phosphatidylcholine optionally in combination with phosphatidylethanolamine.

19. The composition of claim **1**, which is aqueous and wherein the liposomes are suspended in a continuous aqueous medium.

20. The composition of claim **1**, which is in dry form.

21. The composition of claim **1**, additionally comprising one or more sugars.

22. The composition of claim **1**, which is a pharmaceutical composition and comprises a pharmaceutically acceptable diluent or excipient.

23. The composition of claim **1**, wherein at least one and preferably each polysaccharide antigen is entrapped in the intravesicular space of the liposomes.

24. The composition of claim **1**, wherein the protein carrier is entrapped in the intravesicular space.

25. The composition of claim **1**, wherein the liposomes have an average diameter in the range 50-700 nm.

26. A method of eliciting an immune response in an animal by administering to an animal in need of such response a composition of claim **1**, whereby an immune response is elicited.

27. The method of claim **26**, wherein the immune response comprises production of IgG, IgM or IgA, specific to the polysaccharide antigen.

28. The method of claim **26**, wherein the composition is administered sub-cutaneously, intravenously, intraperito-

neally, intramuscularly, intranasally, by inhalation, intravaginally, buccally or orally, preferably sub-cutaneously.

29. The method of claim **26**, wherein the composition is administered in a unit dosage comprising 1-30 μg protein.

30. The method of claim **26**, wherein the immune response is a protective response against infection by the microbe from which the polysaccharide antigen is derived.

31. The method of claim **26**, wherein the animal is a human.

32-34. (canceled)

35. The method for forming a liposomal composition of claim **1**, comprising (i) mixing empty liposomes with two or more polysaccharide antigens and protein carrier; and (ii) drying the mixture from step (i).

36. The method of claim **35**, which further comprises the step (iii) of rehydrating the mixture from step (ii).

37. The method of claim **36**, in which separate aliquots of empty liposomes are mixed with each polysaccharide antigen and protein carrier and, after step (iii) the liposome suspensions are blended to form a multivalent antigen composition.

38. The method of claim **35**, wherein in step (i), a sugar is present in the mixture of polysaccharide antigen, protein carrier, and liposomes.

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