(54) Title: CARRIER MOLECULE COMPRISING A SPR0096 AND A SPR2021 ANTIGEN

FIG. 3

(57) Abstract: The invention provides a conjugate comprising an antigen and a carrier molecule, wherein the carrier molecule comprises a spr0096 antigen and a spr2021 antigen. spr0096 and spr2021 are Streptococcus pneumoniae antigens. The conjugate may be used in a method for raising an immune response in a mammal, the method comprising administering the conjugate to the mammal. Also provided are pharmaceutical compositions, particularly vaccines, comprising the conjugate.
This application claims the benefit of U.S. Provisional Application Serial No. 61/556,456, filed on 7th November 2011; and U.S. Provisional Application Serial No. 61/566,407, filed on 2nd December 2011, both of which are incorporated by reference herein in their entirety.

5 TECHNICAL FIELD

The invention relates to conjugates of antigens and carrier molecules, and vaccines comprising these conjugates. The antigens are typically saccharides.

BACKGROUND OF THE INVENTION

The use of conjugation to carrier proteins in order to enhance the immunogenicity of saccharide antigens is well known [e.g. reviewed in refs. 1 to 9 etc.] and is used in particular for paediatric vaccines [10]. Three widely used carrier proteins in present-day vaccines are tetanus toxoid (TT), diphtheria toxoid (DT) and the diphtheria toxoid variant, CRM197. These proteins have been used as carriers for various saccharides, particularly meningococcal capsular saccharides (see, for example, the use of TT as carrier for saccharides derived from N.meningitidis serogroups A, C, W135 and Y in ref. 11; and DT and CRM197 as carriers for the same saccharides in refs. 12 and 13 respectively). Concerns have been raised about the overuse of these carrier proteins in vaccines [see, for example, ref. 14], with various alternative carriers being suggested (e.g. protein D from H.influenzae in ref. 15). However, many alternative carrier proteins are not as effective as TT, DT and/or CRM197. Accordingly, there remains a need to find alternative and/or better carrier proteins.

10 It is therefore an object of the invention to provide further and better carrier proteins, particularly carrier proteins for meningococcal capsular saccharides. The carrier proteins may be used in conjugates to induce protective and/or therapeutic immune responses against infections or drugs.

SUMMARY OF THE INVENTION

The inventors have found that proteins comprising two specific Streptococcus pneumoniae antigens, a spr0096 antigen and a spr2021 antigen, are effective carriers. These carriers are versatile and may be conjugated to various antigens, particularly saccharides e.g. from pathogenic organisms. The resultant conjugates may be more immunogenic than conjugates based on currently used carrier proteins, e.g. CRM197. Moreover, they may provide higher levels of protective immunity against pathogens from which the saccharides are derived.

25 The invention therefore provides a conjugate comprising an antigen and a carrier molecule, wherein the carrier molecule comprises a spr0096 antigen and a spr2021 antigen. The carrier molecule typically comprises the spr0096 antigen and the spr2021 antigen as a single polypeptide chain (a “hybrid” polypeptide). Typically, the antigen is a saccharide. The saccharide may be any saccharide, particularly a saccharide from a pathogenic organism. For example, the saccharide may be a capsular saccharide from N.meningitidis, a glucan or a capsular saccharide from S.pneumoniae. When the saccharide is a capsular saccharide from N.meningitidis, it is typically from one of the
following meningococcal serogroups: A, C, W135 and Y. When the saccharide is a glucan, it is typically a laminarin. When the saccharide is a capsular saccharide from \textit{S.pneumoniae}, it is typically from one of the following pneumococcal serotypes: 1, 2, 3, 4, 5, 6A, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F. However, in some embodiments, the saccharide is not a capsular saccharide from \textit{S.pneumoniae}.

The present invention also relates to pharmaceutical compositions comprising a conjugate of the invention in combination with a pharmaceutically acceptable carrier.

The present invention further relates to methods for raising an immune response in a mammal, comprising administering a conjugate or pharmaceutical composition of the invention to the mammal.

The present invention further relates to carrier molecules which have been modified to include non-natural amino acids.

**BRIEF DESCRIPTION OF DRAWINGS**

Figure 1 provides the repeating units of representative bacterial saccharides for use in the invention.

Figure 2 compares the immunogenicity of laminarin conjugated to various pneumococcal proteins and a reference carrier, CRM197.

Figure 3 compares the immunogenicity of a laminarin conjugate of the invention with a reference CRM197 conjugate.

Figure 4 compares the immunogenicity of various pneumococcal and meningococcal saccharide conjugates of the invention with reference CRM197 conjugates.

Figure 5 compares the immunogenicity of a pneumococcal conjugate of the invention with a reference CRM197 conjugate.

Figure 6 compares the effects of a pneumococcal conjugate of the invention with a reference CRM197 conjugate in a model of protective immunity against pneumococcus serotype 5 infection.

Figure 7 compares the effects of a pneumococcal conjugate of the invention, a reference CRM197 conjugate, and the pneumococcal saccharide and carrier alone and together, in a model of protective immunity against pneumococcus serotype 5 infection.

Figures 8-10 compare the immunogenicity of a meningococcal serogroup A conjugate of the invention with a reference CRM197 conjugate, alone and in combination with other meningococcal conjugates. In Figures 9 and 10, SBA titers are given above each bar.

Figure 11 compares the immunogenicity of a meningococcal serogroup C conjugate of the invention with a reference CRM197 conjugate, alone and in combination with other meningococcal conjugates.
Figure 12 compares the immunogenicity of a meningococcal serogroup W135 conjugate of the invention with a reference CRM197 conjugate, alone and in combination with other meningococcal conjugates.

Figure 13 compares the immunogenicity of a meningococcal serogroup Y conjugate of the invention with a reference CRM197 conjugate, alone and in combination with other meningococcal conjugates.

Figure 14 compares the immunogenicity of a meningococcal serogroup C conjugate of the invention with a reference CRM197 conjugate and a spr1416 conjugate.

Figure 15 compares the T-cell response to a meningococcal serogroup C conjugate of the invention with a reference CRM197 conjugate and a spr1416 conjugate.

Figure 16 shows the mass spectrometry trace of a carrier of the invention which has been expressed in a host cell such that l-homoallylglycine residues have been incorporated into the protein at the positions normally comprising methionine (SEQ ID NO: 20 vs. SEQ ID NO: 9).

Figure 17 compares the immunogenicity of a meningococcal serogroup A conjugate of the invention with reference CRM197 conjugates, alone and in combination with other meningococcal conjugates.

Figure 18 compares the immunogenicity of the other meningococcal conjugates in the study of Figure 17.

**DETAILED DESCRIPTION OF THE INVENTION**

The invention involves a conjugate comprising an antigen and a carrier molecule, wherein the carrier molecule comprises a spr0096 antigen and a spr2021 antigen. The features of this conjugate are described in detail below.

The invention also involves carrier molecules which have been modified to incorporate non-natural amino acids. Exemplary modifications and carrier proteins are also described in detail below.

**The carrier molecule**

The carrier molecule comprises a spr0096 antigen and a spr2021 antigen. Typically, the carrier molecule comprises the spr0096 antigen and the spr2021 antigen as a single polypeptide chain (a “hybrid” polypeptide).

**spr0096 antigen**

The original 'spr0096' polypeptide sequence was annotated in reference 16 as 'hypothetical protein' (see GI:15902140). For reference purposes, the amino acid sequence of full length spr0096 as found in the R6 strain is given as SEQ ID NO: 1 herein.

The spr0096 antigen of the invention comprises at least one CD4+ T cell epitope. CD4+ T cells help B lymphocytes to produce antibodies against antigens [17]. T-cell epitopes can be identified empirically (e.g. using PEPSCAN [18,19] or similar methods), or they can be predicted (e.g. using the Jameson-Wolf antigenic index [20], matrix-based approaches [21], TEPITOPE [22], neural
Preferred spr0096 antigens for use with the invention comprise an amino acid sequence: (a) having 50% or more identity (e.g. 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 1; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 1, wherein 'n' is 7 or more (e.g. 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). These spr0096 polypeptides include variants of SEQ ID NO: 1 (e.g. SEQ ID NO: 2; see below). Preferred fragments of (b) comprise at least one CD4+ T cell epitope from SEQ ID NO: 1. Other preferred fragments lack one or more amino acids (e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 1 while retaining at least one CD4+ T cell epitope of SEQ ID NO: 1. Other fragments omit one or more protein domains. One suitable fragment is SEQ ID NO: 14, which omits the natural leader peptide sequence. The spr0096 antigen may consist of a single CD4+ T cell epitope from SEQ ID NO: 1.

A variant form of spr0096, with an insert near its C-terminus relative to SEQ ID NO: 1, is SEQ ID NO: 2 herein. The use of this variant for immunisation is reported in reference 31 (SEQ ID NO: 150 therein), where it is annotated as a LysM domain protein. Thus a spr0096 antigen for use with the invention may comprise an amino acid sequence: (a) having 50% or more identity (e.g. 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 2; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 2, wherein 'n' is 7 or more (e.g. 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). These polypeptides include variants of SEQ ID NO: 2. Preferred fragments of (b) comprise at least one CD4+ T cell epitope from SEQ ID NO: 2. Other preferred fragments lack one or more amino acids (e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 2 while retaining at least one CD4+ T cell epitope of SEQ ID NO: 2. Other fragments omit one or more protein domains. One suitable fragment is SEQ ID NO: 15, which omits the natural leader peptide sequence. Immunogenic fragments of SEQ ID NO: 2 are identified in table 1 of reference 31. The spr0096 antigen may consist of a single CD4+ T cell epitope from SEQ ID NO: 2.

A spr0096 antigen may be used in the form of a dimer, e.g. a homodimer.

**spr2021 antigen**

The original 'spr2021' polypeptide sequence was annotated in reference 16 as 'General stress protein GSP-781' (see GI:15904062). For reference purposes, the amino acid sequence of full length spr2021 as found in the R6 strain is given as SEQ ID NO: 3 herein.
The spr2021 antigen of the invention comprises at least one CD4+ T cell epitope.

Preferred spr2021 antigens for use with the invention comprise an amino acid sequence: (a) having 50% or more identity (e.g. 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 3; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 3, wherein 'n' is 7 or more (e.g. 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). These spr2021 polypeptides include variants of SEQ ID NO: 3. Preferred fragments of (b) comprise at least one CD4+ T cell epitope from SEQ ID NO: 3. Other preferred fragments lack one or more amino acids (e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 3 while retaining at least one CD4+ T cell epitope of SEQ ID NO: 3. Other fragments omit one or more protein domains. One suitable fragment is SEQ ID NO: 4, which omits the natural leader peptide sequence. The spr0096 antigen may consist of a single CD4+ T cell epitope from SEQ ID NO: 3.

Reference 31 annotates spr2021 as a secreted 45kDa protein with homology to GbpB and discloses its use as an immunogen (SEQ ID NO: 243 therein; SP2216). Immunogenic fragments of spr2021 are identified in table 1 of reference 31 (page 73). Another useful fragment of spr2021 is disclosed as SEQ ID NO: 1 of reference 32 (amino acids 28-278 of SEQ ID NO: 3 herein).

**Hybrid polypeptide**

Typically, the spr0096 antigen and spr2021 antigen are expressed as a single polypeptide chain (a ‘hybrid’ polypeptide). Hybrid polypeptides can be represented by the formula NH2-A-{X-L}n-B-COOH, wherein: A is an optional N-terminal amino acid sequence; B is an optional C-terminal amino acid sequence; n is an integer of 2 or more (e.g. 2, 3, 4, 5, 6, etc.); each X is an amino acid sequence of an spr0096 antigen or an spr2021 antigen (as described above), wherein at least one X is an spr0096 antigen and at least one X is an spr2021 antigen; and L is an optional linker amino acid sequence. Usually n is 2. When n is 2, X1 is usually an spr0096 antigen and X2 is usually an spr2021 antigen. When n is more than 2, each spr0096 antigen (when more than one is present) may be the same or different and each spr2021 antigen (when more than one is present) may be the same or different.

The spr0096 antigen or spr2021 antigen that is the amino acid sequence of each X is as defined above. Where these antigens are defined in terms of (a) having 50% or more identity (e.g. 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to a given sequence; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of a given sequence, wherein 'n' is 7 or more (e.g. 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more), the level of identity in (a) and the value of 'n' in (b) may be the same for each X.
The leader peptide sequence in the wild-type form of each -X- moiety may be included or omitted in the hybrid protein. In some embodiments, the leader peptides will be deleted except for that of the -X- moiety located at the N-terminus of the hybrid protein i.e. the leader peptide of X₁ will be retained, but the leader peptides of X₂ … Xₙ will be omitted. This is equivalent to deleting all leader peptides and using the leader peptide of X₁ as moiety -A-

For each n instances of {-X-L-}, linker amino acid sequence -L- may be present or absent. For instance, when n=2 the hybrid may be NH₂-X₁-L₁-X₂-L₂-COOH, NH₂-X₁-X₂-COOH, NH₂-X₁-L₁-X₂-COOH, NH₂-X₁-X₂-L₂-COOH, etc. Linker amino acid sequence(s) -L- will typically be short (e.g. 20 or fewer amino acids i.e. 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1). Examples comprise short peptide sequences which facilitate cloning, poly-glycine linkers (i.e. comprising Glyᵥ where n = 2, 3, 4, 5, 6, 7, 8, 9, 10 or more), and histidine tags (i.e. Hisᵦ where n = 3, 4, 5, 6, 7, 8, 9, 10 or more). Other suitable linker amino acid sequences will be apparent to those skilled in the art. A useful linker is GSGGGG (SEQ ID NO:5) or GSGSGGGG (SEQ ID NO:6), with the Gly-Ser dipeptide being formed from a BamHI restriction site, thus aiding cloning and manipulation, and the (Gly)₄ tetrapeptide being a typical poly-glycine linker. Other suitable linkers, particularly for use as the final Lᵦ are a Leu-Glu dipeptide or SEQ ID NO: 7.

-A- is an optional N-terminal amino acid sequence. This will typically be short (e.g. 40 or fewer amino acids i.e. 40, 39, 38, 37, 36, 35, 34, 33, 32, 31, 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1). Examples include leader sequences to direct protein trafficking, or short peptide sequences which facilitate cloning or purification (e.g. histidine tags i.e. Hisᵦ where n = 3, 4, 5, 6, 7, 8, 9, 10 or more). Other suitable N-terminal amino acid sequences will be apparent to those skilled in the art. If X₁ lacks its own N-terminus methionine, -A- is preferably an oligopeptide (e.g. with 1, 2, 3, 4, 5, 6, 7 or 8 amino acids) which provides a N-terminus methionine e.g. Met-Ala-Ser, or a single Met residue.

-B- is an optional C-terminal amino acid sequence. This will typically be short (e.g. 40 or fewer amino acids i.e. 39, 38, 37, 36, 35, 34, 33, 32, 31, 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1). Examples include sequences to direct protein trafficking, short peptide sequences which facilitate cloning or purification (e.g. comprising histidine tags i.e. Hisᵦ where n = 3, 4, 5, 6, 7, 8, 9, 10 or more, such as SEQ ID NO: 8), or sequences which enhance protein stability. Other suitable C-terminal amino acid sequences will be apparent to those skilled in the art.

Examples of hybrids include polypeptides that comprise an amino acid sequence of spr0096-spr2021 (e.g. SEQ ID NO: 9) or spr2021-spr0096 (e.g. SEQ ID NO: 10). The hybrid may also comprise an amino acid sequence having 50% or more identity (e.g. 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 9 or 10. Typically, the hybrid comprises an amino acid sequence of SEQ ID NO: 9. The hybrid may also
comprise an amino acid sequence having 50% or more identity (e.g. 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 9.

In particular embodiments, the carrier molecule comprises (a) one or more (e.g. 1, 2, 3, 4, 5, etc.) CD4\(^+\) T cell epitopes from SEQ ID NO: 2; and (b) one or more (e.g. 1, 2, 3, 4, 5, etc.) CD4\(^+\) T cell epitopes from SEQ ID NO: 3.

**Carrier molecules modified to incorporate non-natural amino acids**

The invention also involves carrier molecules which have been modified to incorporate non-natural amino acids. The non-natural amino acid may be used to conjugate the carrier molecule to another molecule.

In some alternatives, the carrier molecule comprises one or more (e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 etc.) non-natural amino acids. The non-natural amino acid may have a functional group with a reaction profile that is different to the functional groups available to react in proteins composed of the canonical amino acids (e.g. the amino group of lysine or the sulphydryl group of cysteine). This in turn means that chemoselective reactions allow site-selective conjugations to be performed at pre-determined sites where a non-natural amino acid has been incorporated into the protein.

In particular embodiments, the carrier molecule comprises one or more L-homoallylglycine (HAG) residues. Typically HAG residues are substituted in place of the methionine residues in the sequence. HAG, chemically known as L-2-amino-5-hexenoic acid, is an analogue of methionine, and contains a reactive alkene site. HAG can substitute for methionine in both the initiation and elongation steps of protein synthesis. HAG has an olefinic side-chain which has a different reaction profile to the functional groups found in canonical amino acids, reacting through a thyl-ene mechanism.

In other embodiments, the carrier molecule may be modified to include other non-natural amino acids which permit site-selective conjugations to be performed at pre-determined sites. For example, the carrier molecule may be modified so that one or more (e.g. 1, 2, 3, 4, 5, etc.) p-acetylphenylalanine residues are included in its sequence. This amino acid has a keto functional group, which is not present in any of the canonical amino acids, and therefore the amino acid can be reacted specifically with hydrazines, alkoxyamines and semicarbazides under mild aqueous conditions to produce hydrazine, oxime and semicarbazone linkages. Other amino acids with keto functional groups include m-acetylphenylalanine and p-benzoylephenylalanine and these residues may be used in the same manner.

In other embodiments, the carrier molecule may be modified to include an azide group (which also does not occur in the canonical amino acids), for example by incorporation of one or more (e.g. 1, 2, 3, 4, 5, etc.) p-azidophenylalanine residues. The azide group can react with an acetylene group on the conjugation partner through a copper (I) catalysed [2+3] cycloaddition reaction. Conversely, it is possible to engineer the non-naturally occurring acetylene group into the carrier protein by
incorporation of one or more (e.g. 1, 2, 3, 4, 5, etc.) \( p \)-propargyloxyphenylalanine residues, which can then be reacted through the same mechanism with an azide group on the conjugation partner.

In yet further embodiments, the carrier molecule may be modified to include one or more (e.g. 1, 2, 3, 4, 5, etc.) phenylselenocysteine residues. Treatment of this residue with hydrogen peroxide allows its conjugation to thiol groups.

In exemplary modified carrier molecules of the invention, the spr0096 antigen may comprise an amino acid sequence: (a) having 50% or more identity (e.g. 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 1; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 1, wherein 'n' is 7 or more (e.g. 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more), wherein one or more of the methionine residues in the polypeptide is replaced with HAG. For example, the carrier molecule may have the sequence set out in SEQ ID NO: 16.

A variant form of spr0096, with an insert near its C-terminus relative to SEQ ID NO: 1, is SEQ ID NO: 2 herein. Thus a spr0096 antigen for use with the invention may comprise an amino acid sequence: (a) having 50% or more identity (e.g. 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 2; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 2, wherein 'n' is 7 or more (e.g. 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more), wherein one or more of the methionine residues in the polypeptide is replaced with HAG. For example, the carrier molecule may have the sequence set out in SEQ ID NO: 17. In other or the same examples of modified carrier molecules of the invention, the spr2021 antigen may comprise an amino acid sequence: (a) having 50% or more identity (e.g. 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 3; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 3, wherein 'n' is 7 or more (e.g. 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more), wherein one or more of the methionine residues in the polypeptide is replaced with HAG. In some embodiments two or more, three or more, or four or more of the methionine residues in the polypeptide are replaced with HAG. For example, the carrier molecule may have the sequence set out in SEQ ID NO: 18.

A variant form of spr2021 is SEQ ID NO: 4, which omits the natural leader peptide sequence. Thus a spr2021 antigen for use with the invention may comprise an amino acid sequence: (a) having 50% or more identity (e.g. 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 4; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 4, wherein 'n' is 7 or more (e.g. 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more), wherein one or more of the methionine residues in the polypeptide is replaced with HAG. For example, the carrier molecule may have the sequence set out in SEQ ID NO: 19.
Further examples of modified carrier molecules include hybrid polypeptides as defined above, wherein one or more of the methionine residues in the polypeptide is replaced with HAG. For example, the hybrid polypeptide may comprise an amino acid sequence of spr0096-spr2021 (e.g. SEQ ID NO: 9) or spr2021-spr0096 (e.g. SEQ ID NO: 10), or an amino acid sequence having 50% or more identity (e.g. 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 9 or 10, wherein one or more of the methionine residues in the polypeptide is replaced with HAG. In some embodiments two or more, or three or more of the methionine residues in the polypeptide are replaced with HAG. For example, the carrier molecule may have the sequence set out in SEQ ID NO: 20 or 21. In particular embodiments, the carrier molecule comprises (a) one or more (e.g. 1, 2, 3, 4, 5, etc.) CD4\(^+\) T cell epitopes from SEQ ID NO: 2; and/or (b) one or more (e.g. 1, 2, 3, 4, 5, etc.) CD4\(^+\) T cell epitopes from SEQ ID NO: 3.

These techniques can also be applied to other known non-natural amino acids, and further to other carrier molecules. Accordingly in the above embodiments, the carrier molecule may be any one of these other carrier molecules. Preferred carrier molecules include bacterial toxins, such as diphtheria or tetanus toxins, or toxoids or mutants thereof. These are commonly used in conjugate vaccines. The CRM\(_{197}\) diphtheria toxin mutant is particularly preferred [33]. Fragment C of tetanus toxoid may also be used [34]. Other carrier molecules include antigens, such as spr0096 or spr2021 as described above. Further suitable carrier molecules include the \textit{N.meningitidis} outer membrane protein complex [35], synthetic peptides [36,37], heat shock proteins [38,39], pertussis proteins [40,41], cytokines [42], lymphokines [150], hormones [150], growth factors [150], artificial proteins comprising multiple human CD4\(^+\) T cell epitopes from various pathogen-derived antigens [43] such as N19 [44], protein D from \textit{H.influenzae} [45-47], pneumolysin [48] or its non-toxic derivatives [49], pneumococcal surface protein PspA [50], iron-uptake proteins [51], toxin A or B from \textit{C.difficile} [52], recombinant \textit{Pseudomonas aeruginosa} exoprotein A (rEPA) [53], etc.

The antigen

The antigen is typically a saccharide. When the antigen is a saccharide, the saccharide may be any saccharide, particularly a saccharide from a pathogenic organism. Exemplary saccharides for use in the invention are described below. In particular, the saccharide may be a bacterial saccharide, \textit{e.g.} a bacterial capsular saccharide. Representative bacterial saccharides are described in Figure 1.

The saccharides may be used in the form of oligosaccharides. These are conveniently formed by fragmentation of purified polysaccharide (\textit{e.g.} by hydrolysis), which will usually be followed by purification of the fragments of the desired size. Saccharides may be purified from natural sources. As an alternative to purification, saccharides may be obtained by total or partial synthesis.

When the antigen is not a saccharide, it may be any other antigen, \textit{i.e.} any immunogen or hapten. Conjugates of the invention may elicit an immune response against a hapten conjugated to the carrier molecule. The hapten may for example be a drug of abuse [54]. Examples include, but are not limited to, opiates, marijuana, amphetamines, cocaine, barbiturates, glutethimide, methyprylon,
chloral hydrate, methaqualone, benzodiazepines, LSD, nicotine, anticholinergic drugs, antipsychotic drugs, tryptamine, other psychomimetic drugs, sedatives, phencyclidine, psilocybine, volatile nitrite, and other drugs inducing physical and/or psychological dependence.

*N. meningitidis* capsular saccharides

The saccharide may be a bacterial capsular saccharide. Exemplary bacterial capsular saccharides include those from *N. meningitidis*. Based on the organism’s capsular polysaccharide, various serogroups of *N. meningitidis* have been identified, including A, B, C, H, I, K, L, 29E, W135, X, Y & Z. The saccharide in the invention may be any of these serogroups. Typically, the saccharide is from one of the following meningococcal serogroups: A, C, W135 and Y.

The capsular saccharides will generally be used in the form of oligosaccharides. These are conveniently formed by fragmentation of purified capsular polysaccharide (e.g. by hydrolysis), which will usually be followed by purification of the fragments of the desired size.

Fragmentation of polysaccharides is typically performed to give a final average degree of polymerisation (DP) in the oligosaccharide of less than 30 (e.g. between 10 and 20, preferably around 10 for serogroup A; between 15 and 25 for serogroups W135 and Y, preferably around 15-20; between 12 and 22 for serogroup C; etc.). DP can conveniently be measured by ion exchange chromatography or by colorimetric assays [55].

If hydrolysis is performed, the hydrolysate will generally be sized in order to remove short-length oligosaccharides [56]. This can be achieved in various ways, such as ultrafiltration followed by ion-exchange chromatography. Oligosaccharides with a degree of polymerisation of less than or equal to about 6 are preferably removed for serogroup A, and those less than around 4 are preferably removed for serogroups W135 and Y.

Chemical hydrolysis of saccharides generally involves treatment with either acid or base under conditions that are standard in the art. Conditions for depolymerisation of capsular saccharides to their constituent monosaccharides are known in the art. One depolymerisation method involves the use of hydrogen peroxide [57]. Hydrogen peroxide is added to a saccharide (e.g. to give a final H₂O₂ concentration of 1%), and the mixture is then incubated (e.g. at around 55°C) until a desired chain length reduction has been achieved. The reduction over time can be followed by removing samples from the mixture and then measuring the (average) molecular size of saccharide in the sample. Depolymerization can then be stopped by rapid cooling once a desired chain length has been reached

**Serogroups C, W135 and Y**

Techniques for preparing capsular polysaccharides from meningococci have been known for many years, and typically involve a process comprising the steps of polysaccharide precipitation (e.g. using a cationic detergent), ethanol fractionation, cold phenol extraction (to remove protein) and ultracentrifugation (to remove LPS) [e.g. see ref. 58].
A more preferred process [59] involves polysaccharide precipitation followed by solubilisation of the precipitated polysaccharide using a lower alcohol. Precipitation can be achieved using a cationic detergent such as tetrabutylammonium and cetyltrimethylammonium salts (e.g. the bromide salts), or hexadimethrine bromide and myristytrimethylammonium salts. Cetyltrimethylammonium bromide ('CTAB') is particularly preferred [60]. Solubilisation of the precipitated material can be achieved using a lower alcohol such as methanol, propan-1-ol, propan-2-ol, butan-1-ol, butan-2-ol, 2-methyl-propan-1-ol, 2-methyl-propan-2-ol, diols, etc., but ethanol is particularly suitable for solubilising CTAB-polysaccharide complexes. Ethanol may be added to the precipitated polysaccharide to give a final ethanol concentration (based on total content of ethanol and water) of between 50% and 95%.

After re-solubilisation, the polysaccharide may be further treated to remove contaminants. This is particularly important in situations where even minor contamination is not acceptable (e.g. for human vaccine production). This will typically involve one or more steps of filtration e.g. depth filtration, filtration through activated carbon may be used, size filtration and/or ultrafiltration.

Once filtered to remove contaminants, the polysaccharide may be precipitated for further treatment and/or processing. This can be conveniently achieved by exchanging cations (e.g. by the addition of calcium or sodium salts).

After purification, the capsular saccharides are conjugated to carrier proteins as described below.

Further and alternative methods for purification and conjugation of meningococal saccharides are disclosed in references 57 & 61.

As an alternative to purification, capsular saccharides of the present invention may be obtained by total or partial synthesis e.g. Hib synthesis is disclosed in ref. 62, and MenA synthesis in ref. 63.

The saccharide may be chemically modified e.g. it may be O-acetylated or de-O-acetylated. Any such de-O-acetylation or hyper-acetylation may be at specific positions in the saccharide. For instance, most serogroup C strains have O-acetyl groups at position C-7 and/or C-8 of the sialic acid residues, but about 15% of clinical isolates lack these O-acetyl groups [64,65]. The acetylation does not seem to affect protective efficacy (e.g. unlike the Menjugate™ product, the NeisVac-C™ product uses a de-O-acetylated saccharide, but both vaccines are effective). The serogroup W135 saccharide is a polymer of sialic acid-galactose disaccharide units. The serogroup Y saccharide is similar to the serogroup W135 saccharide, except that the disaccharide repeating unit includes glucose instead of galactose. Like the serogroup C saccharides, the MenW135 and MenY saccharides have variable O-acetylation, but at sialic acid 7 and 9 positions [66]. Any such chemical modifications preferably take place before conjugation, but may alternatively or additionally take place during conjugation.

Saccharides from different serogroups are preferably purified separately, and may then be combined, either before or after conjugation.
Serogroup A

Conjugates of the invention may include a serogroup A capsular saccharide antigen. The saccharide can be purified and conjugated in the same way as for serogroups C, W135 and Y (see above), although it is structurally different – whereas the capsules of serogroups C, W135 and Y are based around sialic acid (N-acetyl-neuraminic acid, NeuAc), the capsule of serogroup A is based on N-acetyl-mannosamine, which is the natural precursor of sialic acid. The serogroup A saccharide is particularly susceptible to hydrolysis, and its instability in aqueous media means that (a) the immunogenicity of liquid vaccines against serogroup A declines over time, and (b) quality control is more difficult, due to release of saccharide hydrolysis products into the vaccine.

Native MenA capsular saccharide is a homopolymer of (α1→6)-linked N-acetyl-D-mannosamine-1-phosphate, with partial O-acetylation at C3 and C4. The principal glycosidic bond is a 1-6 phosphodiester bond involving the hemiacetal group of C1 and the alcohol group of C6 of the D-mannosamine. The average chain length is 93 monomers. It has the following formula:

A modified saccharide antigen has been prepared which retains the immunogenic activity of the native serogroup A saccharide but which is much more stable in water. Hydroxyl groups attached at carbons 3 and 4 of the monosaccharide units are replaced by a blocking group [refs. 67 and 68].

The number of monosaccharide units having blocking groups in place of hydroxyls can vary. For example, all or substantially all the monosaccharide units may have blocking groups. Alternatively, at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80% or 90% of the monosaccharide units may have blocking groups. At least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 monosaccharide units may have blocking groups.

Likewise, the number of blocking groups on a monosaccharide unit may vary. For example, the number of blocking groups on any particular monosaccharide unit may be 1 or 2.
The terminal monosaccharide unit may or may not have a blocking group instead of its native hydroxyl. It is preferred to retain a free anomeric hydroxyl group on a terminal monosaccharide unit in order to provide a handle for further reactions (e.g., conjugation). Anomeric hydroxyl groups can be converted to amino groups (–NH₂ or –NH-E, where E is a nitrogen protecting group) by reductive amination (using, for example, NaBH₃CN/NH₄Cl), and can then be regenerated after other hydroxyl groups have been converted to blocking groups.

Blocking groups to replace hydroxyl groups may be directly accessible via a derivatizing reaction of the hydroxyl group i.e. by replacing the hydrogen atom of the hydroxyl group with another group. Suitable derivatives of hydroxyl groups which act as blocking groups are, for example, carbamates, sulfonates, carbonates, esters, ethers (e.g. silyl ethers or alkyl ethers) and acetals. Some specific examples of such blocking groups are allyl, Aloc, benzyl, BOM, t-butyl, trityl, TBS, TBDPS, TES, TMS, TIPS, PMB, MEM, MOM, MTM, THP, etc. Other blocking groups that are not directly accessible and which completely replace the hydroxyl group include C₁₋₁₂ alkyl, C₃₋₁₂ alkyl, C₅₋₁₂ aryl, C₅₋₁₂ aryl-C₁₋₆ alkyl, NR₁R₂ (R¹ and R² are defined in the following paragraph), H, F, Cl, Br, CO₂H, CO₂(C₁₋₆ alkyl), CN, CF₃, CCl₃, etc.

Typical blocking groups are of the formula: –O–X–Y or –OR³ wherein: X is C(O), S(O) or SO₂; Y is C₁₋₁₂ alkyl, C₁₋₁₂ alkoxy, C₃₋₁₂ cycloalkyl, C₅₋₁₂ aryl or C₅₋₁₂ aryl-C₁₋₆ alkyl, each of which may optionally be substituted with 1, 2 or 3 groups independently selected from F, Cl, Br, CO₂H, CO₂(C₁₋₆ alkyl), CN, CF₃ or CCl₃; or Y is NR¹R²; R¹ and R² are independently selected from H, C₁₋₁₂ alkyl, C₃₋₁₂ cycloalkyl, C₅₋₁₂ aryl, C₅₋₁₂ aryl-C₁₋₆ alkyl; or R¹ and R² may be joined to form a C₃₋₁₂ saturated heterocyclic group; R³ is C₁₋₁₂ alkyl or C₃₋₁₂ cycloalkyl, each of which may optionally be substituted with 1, 2 or 3 groups independently selected from F, Cl, Br, CO₂(C₁₋₆ alkyl), CN, CF₃ or CCl₃; or R³ is C₅₋₁₂ aryl or C₅₋₁₂ aryl-C₁₋₆ alkyl, each of which may optionally be substituted with 1, 2, 3, 4 or 5 groups selected from F, Cl, Br, CO₂H, CO₂(C₁₋₆ alkyl), CN, CF₃ or CCl₃. When R³ is C₁₋₁₂ alkyl or C₃₋₁₂ cycloalkyl, it is typically substituted with 1, 2 or 3 groups as defined above. When R¹ and R² are joined to form a C₃₋₁₂ saturated heterocyclic group, it is meant that R¹ and R² together with the nitrogen atom form a saturated heterocyclic group containing any number of carbon atoms between 3 and 12 (e.g. C₃, C₄, C₅, C₆, C₇, C₈, C₉, C₁₀, C₁₁, C₁₂). The heterocyclic group may contain 1 or 2 heteroatoms (such as N, O or S) other than the nitrogen atom. Examples of C₁₋₁₂ saturated heterocyclic groups are pyrroldinyl, piperidinyl, morpholinyl, piperazinyl, imidazolidinyl, azetidinyl and aziridinyl.

Blocking groups -O-X-Y and -OR³ can be prepared from -OH groups by standard derivatizing procedures, such as reaction of the hydroxyl group with an acyl halide, alkyl halide, sulfonyle halide, etc. Hence, the oxygen atom in -O-X-Y is usually the oxygen atom of the hydroxyl group, while the -X-Y group in -O-X-Y usually replaces the hydrogen atom of the hydroxyl group.
Alternatively, the blocking groups may be accessible via a substitution reaction, such as a Mitsonobu-type substitution. These and other methods of preparing blocking groups from hydroxyl groups are well known.

Specific blocking groups for use in the invention are -OC(O)CF₃ [69] and a carbamate group OC(O)NR¹R², where R¹ and R² are independently selected from C₁₋₆ alkyl. Typically, R¹ and R² are both methyl i.e. the blocking group is -OC(O)NMe₂. Carbamate blocking groups have a stabilizing effect on the glycosidic bond and may be prepared under mild conditions.

A particularly preferred blocking group is -OC(O)CH₃ [68]. The proportion of 4- and/or 3-positions in the modified Neisseria meningitidis serogroup A saccharide that have this blocking group may vary. For example, the proportion of 4-positions that have blocking groups may be about 0%, at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or about 100%, with at least 80% and about 100% being preferred. Similarly, the proportion of 3-positions that have blocking groups may be about 0%, at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or about 100%, with at least 80% and about 100% being preferred. Typically, the proportion of 4- and 3-positions that have blocking groups is about the same at each position. In other words, the ratio of 4-positions that have blocking groups to 3-positions that have blocking groups is about 1:1. However, in some embodiments, the proportion of 4-positions that have blocking groups may vary relative to the proportion of 3-positions that have blocking groups. For example, the ratio of 4-positions that have blocking groups to 3-positions that have blocking groups may be 1:20, 1:19, 1:18, 1:17, 1:16, 1:15, 1:14, 1:13, 1:12, 1:11, 1:10, 1:9, 1:8, 1:7, 1:6, 1:5, 1:4, 1:3 or 1:2. Similarly, the ratio of 3-positions that have blocking groups to 4-positions that have blocking groups may be 1:20, 1:19, 1:18, 1:17, 1:16, 1:15, 1:14, 1:13, 1:12, 1:11, 1:10, 1:9, 1:8, 1:7, 1:6, 1:5, 1:4, 1:3 or 1:2.

Typical modified MenA saccharides contain n monosaccharide units, where at least h% of the monosaccharide units do not have –OH groups at both of positions 3 and 4. The value of h is 24 or more (e.g. 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 98, 99 or 100) and is usually 50 or more. The absent –OH groups are blocking groups as defined above.

Other typical modified MenA saccharides comprise monosaccharide units, wherein at least s of the monosaccharide units do not have –OH at the 3 position and do not have –OH at the 4 position. The value of s is at least 1 (e.g. 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 55, 60, 70, 80, 90). The absent –OH groups are blocking groups as defined above.

Suitable modified MenA saccharides for use with the invention have the formula:
wherein

n is an integer from 1 to 100 (particularly an integer from 5 to 25, usually15-25);
T is of the formula (A) or (B):

(A)

(B)

each Z group is independently selected from OH or a blocking group as defined above; and
each Q group is independently selected from OH or a blocking group as defined above;
Y is selected from OH or a blocking group as defined above;
E is H or a nitrogen protecting group;

and wherein more than about 7% (e.g. 8%, 9%, 10% or more) of the Q groups are blocking groups.

In some embodiments, the hydroxyl group attached at carbon 1 in formula (A) is replaced by a
blocking group as defined above. In some embodiments, E in formula (B) is a linker or a carrier
molecule of the invention. When E is a linker, the linker may be covalently bonded to a carrier
molecule of the invention.

Each of the n+2 Z groups may be the same or different from each other. Likewise, each of the n+2 Q
groups may be the same or different from each other. All the Z groups may be OH. Alternatively, at
least 10%, 20, 30%, 40%, 50% or 60% of the Z groups may be OAc. Typically, about 70% of the Z
groups are OAc, with the remainder of the Z groups being OH or blocking groups as defined above.
At least about 7% of Q groups are blocking groups. Typically, at least 10%, 20%, 30%, 40%, 50%,
60%, 70%, 80%, 90% or even 100% of the Q groups are blocking groups.
Glucans

The saccharide may be a glucan. Glucans are glucose-containing polysaccharides found inter alia in fungal cell walls. The α-glucans include one or more α-linkages between glucose subunits, whereas β-glucans include one or more β-linkages between glucose subunits. The glucan used in accordance with the invention includes β linkages, and may contain only β linkages (i.e. no α linkages).

The glucan may comprise one or more β-1,3-linkages and/or one or more β-1,6-linkages. It may also comprise one or more β-1,2-linkages and/or β-1,4-linkages, but normally its only β linkages will be β-1,3-linkages and/or β-1,6-linkages.

The glucan may be branched or linear.

Full-length native β-glucans are insoluble and have a molecular weight in the megadalton range. It is preferred to use soluble glucans in conjugates of the invention. Solubilisation may be achieved by fragmenting long insoluble glucans. This may be achieved by hydrolysis or, more conveniently, by digestion with a glucanase (e.g. a β-1,3-glucanase or a β-1,6-glucanase). As an alternative, short glucans can be prepared synthetically by joining monosaccharide building blocks.

Low molecular weight glucans are preferred, particularly those with a molecular weight of less than 100 kDa (e.g. less than 80, 70, 60, 50, 40, 30, 25, 20, or 15 kDa). It is also possible to use oligosaccharides e.g. containing 60 or fewer (e.g. 59, 58, 57, 56, 55, 54, 53, 52, 51, 50, 49, 48, 47, 46, 45, 44, 43, 42, 41, 40 39, 38, 37, 36, 35, 34, 33, 32, 31, 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4) glucose monosaccharide units. Within this range, oligosaccharides with between 10 and 50 or between 20 and 40 monosaccharide units are preferred.

The glucan may be a fungal glucan. A ‘fungal glucan’ will generally be obtained from a fungus but, where a particular glucan structure is found in both fungi and non-fungi (e.g. in bacteria, lower plants or algae) then the non-fungal organism may be used as an alternative source. Thus the glucan may be derived from the cell wall of a Candida, such as Calbicans, or from Coccidioides immitis, Trichophyton verrucosum, Blastomyces dermatidis, Cryptococcus neoformans, Histoplasma capsulatum, Saccharomyces cerevisiae, Paracoccidioides brasiliensis, or Pythium insidiosum.

There are various sources of fungal β-glucans. For instance, pure β-glucans are commercially available e.g. pustulan (Calbiochem) is a β-1,6-glucan purified from Umbilicaria papulosa. β-glucans can be purified from fungal cell walls in various ways. Reference 70, for instance, discloses a two-step procedure for preparing a water-soluble β-glucan extract from Candida, free from cell-wall mannan, involving NaClO oxidation and DMSO extraction. The resulting product (‘Candida soluble β-D-glucan’ or ‘CSBG’) is mainly composed of a linear β-1,3-glucan with a linear β-1,6-glucan moiety. Similarly, reference 71 discloses the production of GG-zym from Calbicans.

Such glucans from Calbicans, include (a) β-1,6-glucans with β-1,3-glucan lateral chains and an
average degree of polymerisation of about 30, and (b) \( \beta-1,3 \)-glucans with \( \beta-1,6 \)-glucan lateral chains and an average degree of polymerisation of about 4.

In some embodiments of the invention, the glucan is a \( \beta-1,3 \) glucan with some \( \beta-1,6 \) branching, as seen in e.g. laminarins. Laminarins are found in brown algae and seaweeds. The \( \beta(1-3):\beta(1-6) \) ratios of laminarins vary between different sources e.g. it is as low as 3:2 in \textit{Eisenia bicyclis} laminarin, but as high as 7:1 in \textit{Laminaria digitata} laminarin [72]. Thus the glucan used with the invention may have a \( \beta(1-3):\beta(1-6) \) ratio of between 1.5:1 and 7.5:1 e.g. about 2:1, 3:1, 4:1, 5:1, 6:1 or 7:1. Optionally, the glucan may have a terminal mannitol subunit, e.g. a 1,1-\( \alpha \)-linked mannitol residue [73]. The glucan may also comprise mannose subunits.

In other embodiments, the glucan has exclusively or mainly \( \beta-1,3 \) linkages, as seen in curdlan. These glucans may elicit better protection than glucans comprising other linkages, particularly glucans comprising \( \beta-1,3 \) linkages and a greater proportion of \( \beta-1,6 \) linkages. Thus the glucan may be made solely of \( \beta-1,3 \)-linked glucose residues (e.g. linear \( \beta-D \)-glucopyranoses with exclusively 1,3 linkages). Optionally, though, the glucan may include monosaccharide residues that are not \( \beta-1,3 \)-linked glucose residues e.g. it may include \( \beta-1,6 \)-linked glucose residues. The ratio of \( \beta-1,3 \)-linked glucose residues to these other residues should be at least 8:1 (e.g. \( \geq 9:1, \geq 10:1, \geq 11:1, \geq 12:1, \geq 13:1, \geq 14:1, \geq 15:1, \geq 16:1, \geq 17:1, \geq 18:1, \geq 19:1, \geq 20:1, \geq 25:1, \geq 30:1, \geq 35:1, \geq 40:1, \geq 45:1, \geq 50:1, \geq 75:1, \geq 100:1, \text{etc.} \) and/or there are one or more (e.g. \( \geq 1, \geq 2, \geq 3, \geq 4, \geq 5, \geq 6, \geq 7, \geq 8, \geq 9, \geq 10, \geq 11, \geq 12, \text{etc.} \) sequences of at least five (e.g. \( \geq 5, \geq 6, \geq 7, \geq 8, \geq 9, \geq 10, \geq 11, \geq 12, \geq 13, \geq 14, \geq 15, \geq 16, \geq 17, \geq 18, \geq 19, \geq 20, \geq 30, \geq 40, \geq 50, \geq 60, \text{etc.} \) adjacent non-terminal residues linked to other residues only by \( \beta-1,3 \) linkages. By “non-terminal” it is meant that the residue is not present at a free end of the glucan. In some embodiments, the adjacent non-terminal residues may not include any residues coupled to a carrier molecule, linker or other spacer as described below. The presence of five adjacent non-terminal residues linked to other residues only by \( \beta-1,3 \) linkages may provide a protective antibody response, e.g. against \textit{C.albicans}.

In further embodiments, a conjugate may include two different glucans e.g. a first glucan having a \( \beta(1-3):\beta(1-6) \) ratio of between 1.5:1 and 7.5:1, and a second glucan having exclusively or mainly \( \beta-1,3 \) linkages. For instance a conjugate may include both a laminarin glucan and a curdlan glucan.

Where a \( \beta \)-glucan includes both \( \beta-1,3 \) and \( \beta-1,6 \) linkages at a desired ratio and/or sequence then this glucan may be found in nature (e.g. a laminarin), or it may be made artificially. For instance, it may be made by chemical synthesis, in whole or in part. Methods for the chemical synthesis of \( \beta-1,3/\beta-1,6 \) glucans are known, for example from references 74-84. \( \beta \)-glucan including both \( \beta-1,3 \) and \( \beta-1,6 \) linkages at a desired ratio may also be made starting from an available glucan and treating it with a \( \beta-1,6 \)-glucanase (also known as glucan endo-1,6-\( \beta \)-glucosidase, 1,6-\( \beta \)-D-glucan glucanohydrolase, etc.; EC 3.2.1.75) or a \( \beta-1,3 \)-glucanase (such as an exo-1,3-glucanase (EC 3.2.1.58) or an endo-1,3-glucanase (EC 3.2.1.39) until a desired ratio and/or sequence is reached.
When a glucan containing solely β-1,3-linked glucose is desired then β-1,6-glucanase treatment may be pursued to completion, as β-1,6-glucanase will eventually yield pure β-1,3 glucan. More conveniently, however, a pure β-1,3-glucan may be used. These may be made synthetically, by chemical and/or enzymatic synthesis e.g. using a (1→3)-β-D-glucan synthase, of which several are known from many organisms (including bacteria, yeasts, plants and fungi). Methods for the chemical synthesis of β-1,3 glucans are known, for example from references 85-88. As a useful alternative to synthesis, a natural β-1,3-glucan may be used, such as a curdlan (linear β-1,3-glucan from an Agrobacterium previously known as Alcaligenes faecalis var. myxogenes; commercially available e.g. from Sigma-Aldrich catalog C7821) or paramylon (β-1,3-glucan from Euglena). Organisms producing high levels of β-1,3-glucans are known in the art e.g. the Agrobacterium of refs. 89 & 90, or the Euglena gracilis of ref. 91.

Laminarin and curdlan are typically found in nature as high molecular weight polymers e.g. with a molecular weight of at least 100kDa. They are often insoluble in aqueous media. In their natural forms, therefore, they are not well suited to immunisation. Thus the invention may use a shorter glucan e.g. those containing 60 or fewer glucose monosaccharide units (e.g. 59, 58, 57, 56, 55, 54, 53, 52, 51, 50, 49, 48, 47, 46, 45, 44, 43, 42, 41, 40 39, 38, 37, 36, 35, 34, 33, 32, 31, 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4). A glucan having a number of glucose residues in the range of 2-60 may be used e.g. between 10-50 or between 20-40 glucose units. A glucan with 25-30 glucose residues is particularly useful. Suitable glucans may be formed e.g. by acid hydrolysis of a natural glucan, or by enzymatic digestion e.g. with a glucanase, such as a β-1,3-glucanase. A glucan with 11-19, e.g. 13-19 and particularly 15 or 17, glucose monosaccharide units is also useful. In particular, glucans with the following structures (A) or (B) are specifically envisaged for use in the present invention:

(A)

wherein n+2 is in the range of 2-60, e.g. between 10-50 or between 2-40. Preferably, n+2 is in the range of 25-30 or 11-19, e.g. 13-17. The inventors have found that n+2 = 15 is suitable.
wherein \( n \) is in the range of 0-9, \( e.g. \) between 1-7 or between 2-6. Preferably, \( n \) is in the range of 3-4 or 1-3. The inventors have found that \( n = 2 \) is suitable.

In some embodiments, the glucan is a single molecular species. In these embodiments, all of the glucan molecules are identical in terms of sequence. Accordingly, all of the glucan molecules are identical in terms of their structural properties, including molecular weight \( \text{etc.} \) Typically, this form of glucan is obtained by chemical synthesis, \( e.g. \) using the methods described above. For example, reference 86 describes the synthesis of a single \( \beta-1,3 \) linked species. Alternatively, in other embodiments, the glucan may be obtained from a natural glucan, \( e.g. \) a glucan from \( L.\text{digitata}, \text{Agrobacterium} \) or \( E\text{uglena} \) as described above, with the glucan being purified until the required single molecular species is obtained. Natural glucans that have been purified in this way are commercially available. A glucan that is a single molecular species may be identified by measuring the polydispersity (Mw/Mn) of the glucan sample. This parameter can conveniently be measured by SEC-MALLS, for example as described in reference 92. Suitable glucans for use in this embodiment of the invention have a polydispersity of about 1, \( e.g. \) 1.01 or less.

Solubility of natural glucans, such as curdlan, can be increased by introducing ionic groups (\( e.g. \) by sulfation, particularly at O-6 in curdlan). Such modifications may be used with the invention, but are ideally avoided as they may alter the glucan’s antigenicity.

When the saccharide is a glucan, it is typically a laminarin.

**S.pneumoniae capsular saccharides**

As discussed above, the saccharide may also be a bacterial capsular saccharide. Further exemplary bacterial capsular saccharides include those from \( S.\text{pneumoniae} \). However, in some embodiments, the saccharide is not a capsular saccharide from \( S.\text{pneumoniae} \).

When the saccharide is a capsular saccharides from \( S.\text{pneumoniae} \), it is typically from one of the following pneumococcal serotypes: 1, 2, 3, 4, 5, 6A, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F, preferably from 1, 5, 6B, 14, 19F and 23F. Capsular polysaccharides from \( S.\text{pneumoniae} \) comprise repeating oligosaccharide units which may contain up to 8 sugar residues. The oligosaccharide units for the main \( S.\text{pneumoniae} \) serotypes are described in Figure 1 and refs 93 and 94.
**S. agalactiae capsular saccharides**

Further exemplary bacterial capsular saccharides include those from *Streptococcus agalactiae* ("GBS"). The capsular saccharide is covalently linked to the peptidoglycan backbone of GBS, and is distinct from the group B antigen, which is another saccharide that is attached to the peptidoglycan backbone.

The GBS capsular saccharides are chemically related, but are antigenically very different. All GBS capsular saccharides share the following trisaccharide core:

\[ \beta-D-Glc\betaNac(1 \rightarrow 3)\beta-D-Gal\beta(1 \rightarrow 4)\beta-D-Glc \]

The various GBS serotypes differ by the way in which this core is modified. The difference between serotypes Ia and III, for instance, arises from the use of either the GlcNAc (Ia) or the Gal (III) in this core for linking consecutive trisaccharide cores. Serotypes Ia and Ib both have a [\(\alpha-D-Neu\betaNac(2 \rightarrow 3)\beta-D-Gal\beta(1 \rightarrow 4)\beta-D-Glc\beta \)] disaccharide linked to the GlcNAc in the core, but the linkage is either 1\( \rightarrow 4 \) (Ia) or 1\( \rightarrow 3 \) (Ib).

GBS-related disease arises primarily from serotypes Ia, Ib, II, III, IV, V, VI, VII, and VIII, with over 85% being caused by five serotypes: Ia, Ib, III & V. The invention may use a saccharide from one of these four serotypes. The capsular saccharides of each of these four serotypes include: (a) a terminal N-acetyl-neuraminic acid (NeuNAc) residue (commonly referred to as sialic acid), which in all cases is linked 2\( \rightarrow 3 \) to a galactose residue; and (b) a N-acetyl-glucosamine residue (GlcNAc) within the trisaccharide core.

All four saccharides include galactose residues within the trisaccharide core, but serotypes Ia, Ib, II & III also contain additional galactose residues in each repeating unit.

Saccharides used according to the invention may be in their native form, or may have been modified. For example, the saccharide may be shorter than the native capsular saccharide, or may be chemically modified. In particular, the serotype V capsular saccharide used in the invention may be modified as described in refs. 95 and 96. For example, a serotype V capsular saccharide that has been substantially desialylated. Desialylated GBS serotype V capsular saccharide may be prepared by treating purified GBS serotype V capsular saccharide under mildly acidic conditions (*e.g.* 0.1M sulphuric acid at 80°C for 60 minutes) or by treatment with neuraminidase, as described in reference 95. Thus the saccharide used according to the invention may be a substantially full-length capsular polysaccharide, as found in nature, or it may be shorter than the natural length. Full-length polysaccharides may be depolymerised to give shorter fragments for use with the invention *e.g.* by hydrolysis in mild acid, by heating, by sizing chromatography, etc. In particular, the serotype II and/or III capsular saccharides used in the invention may be depolymerised as described in refs. 97 and 98.

The saccharide may be chemically modified relative to the capsular saccharide as found in nature. For example, the saccharide may be de-O-acetylated (partially or fully), de-N-acetylated (partially or
fully), N-propionated (partially or fully), etc. De-acetylation may occur before, during or after conjugation, but preferably occurs before conjugation. Depending on the particular saccharide, de-acetylation may or may not affect immunogenicity. The relevance of O-acetylation on GBS saccharides in various serotypes is discussed in reference 99, and in some embodiments O-acetylation of sialic acid residues at positions 7, 8 and/or 9 is retained before, during and after conjugation e.g. by protection/de-protection, by re-acetylation, etc. However, typically the GBS saccharide used in the present invention has substantially no O-acetylation of sialic acid residues at positions 7, 8 and/or 9. In particular, when the GBS saccharide has been purified by base extraction as described below, then O-acetylation is typically lost. The effect of de-acetylation etc. can be assessed by routine assays.

Capsular saccharides can be purified by known techniques, as described in 100. A typical process involves base extraction, centrifugation, filtration, RNase/DNase treatment, protease treatment, concentration, size exclusion chromatography, ultrafiltration, anion exchange chromatography, and further ultrafiltration. Treatment of GBS cells with the enzyme mutanolysin, which cleaves the bacterial cell wall to free the cell wall components, is also useful.

As an alternative, the purification process described in reference 101 can be used. This involves base extraction, ethanol/CaCl₂ treatment, CTAB precipitation, and re-solubilisation. A further alternative process is described in reference 102.

**S.aureus capsular saccharides**

Further exemplary bacterial capsular saccharides include those from *S.aureus*, particularly the capsular polysaccharides of *S.aureus* type 5 and type 8. The structures of type 5 and type 8 capsular polysaccharides were described in references 103 and 104 as:

**Type 5**

\[
\rightarrow 4)-\beta-D-ManNAc(3OAc)-(1 \rightarrow 4)-\alpha-L-FucNAc(1 \rightarrow 3)-\beta-D-FucNAc-(1 \rightarrow
\]

**Type 8**

\[
\rightarrow 3)-\beta-D-ManNAc(4OAc)-(1 \rightarrow 3)-\alpha-L-FucNAc(1 \rightarrow 3)-\beta-D-FucNAc-(1 \rightarrow
\]

Recent NMR spectroscopy data [105] has led to a revision of these structures to:

**Type 5**

\[
\rightarrow 4)-\beta-D-ManNAc-(1 \rightarrow 4)-\alpha-L-FucNAc(3OAc)-(1 \rightarrow 3)-\beta-D-FucNAc-(1 \rightarrow
\]

**Type 8**

\[
\rightarrow 3)-\beta-D-ManNAc(4OAc)-(1 \rightarrow 3)-\alpha-L-FucNAc(1 \rightarrow 3)-\alpha-D-FucNAc(1 \rightarrow
\]

The polysaccharide may be chemically modified relative to the capsular polysaccharide as found in nature.
For example, the polysaccharide may be de-O-acetylated (partially or fully), de-N-acetylated (partially or fully), N-propionated (partially or fully), etc. De-acetylation may occur before, during or after conjugation, but typically occurs before conjugation. Depending on the particular polysaccharide, de-acetylation may or may not affect immunogenicity e.g. the NeisVac-C™ vaccine uses a de-O-acetylated polysaccharide, whereas Menjugate™ is acetylated, but both vaccines are effective. The effect of de-acetylation etc. can be assessed by routine assays. For example, the relevance of O-acetylation on *S. aureus* type 5 or type 8 capsular polysaccharides is discussed in reference 106. The native polysaccharides are said in this document to have 75% O-acetylation. These polysaccharides induced antibodies to both the polysaccharide backbone and O-acetyl groups.

Polysaccharides with 0% O-acetylation still elicited antibodies to the polysaccharide backbone. Both types of antibody were opsonic against *S. aureus* strains that varied in their O-acetyl content. Accordingly, the type 5 or type 8 capsular polysaccharides used in the present invention may have between 0 and 100% O-acetylation.

The degree of O-acetylation of the polysaccharide can be determined by any method known in the art, for example, by proton NMR (e.g. as described in references 107, 108, 109 or 110). A further method is described in reference 111. Similar methods may be used to determine the degree of N-acetylation of the polysaccharide. O-acetyl groups may be removed by hydrolysis, for example by treatment with a base such as anhydrous hydrazine [112] or NaOH [106]. Similar methods may be used to remove N-acetyl groups. To maintain high levels of O-acetylation on type 5 and/or 8 capsular polysaccharides, treatments that lead to hydrolysis of the O-acetyl groups are minimised, e.g. treatments at extremes of pH.

Capsular polysaccharides can be purified by known techniques, as described in the references herein. A typical process involves phenol-ethanol inactivation of *S. aureus* cells, centrifugation, lysisostaphin treatment, RNase/DNase treatment, centrifugation, dialysis, protease treatment, further dialysis, filtration, precipitation with ethanol/CaCl₂, dialysis, freeze-drying, anion exchange chromatography, dialysis, freeze-drying, size exclusion chromatography, dialysis and freeze-drying [113]. An alternative process involves autoclaving *S. aureus* cells, ultrafiltration of the polysaccharide-containing supernatant, concentration, lyophilisation, treatment with sodium metaperiodate to remove teichoic acid, further ultrafiltration, diafiltration, high performance size exclusion liquid chromatography, dialysis and freeze-drying [114].

The invention is not limited to polysaccharides purified from natural sources, however, and the polysaccharides may be obtained by other methods, such as total or partial synthesis.

**Other bacterial capsular saccharides**

Further exemplary bacterial capsular saccharides include those from *Haemophilus influenzae* Type b, *Salmonella enterica* Typhi Vi and *Clostridium difficile*.
**S. agalactiae carbohydrate**

The invention may also use non-capsular bacterial saccharides. An exemplary non-capsular bacterial saccharide is the *S. pyogenes* GAS carbohydrate (also known as the GAS cell wall polysaccharide, or GASP). This saccharide features a branched structure with an L-rhamnopyranose (Rhap) backbone consisting of alternating alpha-(1→2) and alpha-(1→3) links and D-N-acetylglucosamine (GlcNAC) residues beta-(1→3)-connected to alternating rhamnose rings ([115]).

The GAS carbohydrate will generally be in its native form, but it may have been modified. For example, the saccharide may be shorter than the native GAS carbohydrate, or may be chemically modified.

Thus the saccharide used according to the invention may be a substantially full-length GAS carbohydrate, as found in nature, or it may be shorter than the natural length. Full-length polysaccharides may be depolymerised to give shorter fragments for use with the invention *e.g.* by hydrolysis in mild acid, by heating, by sizing chromatography, *etc.* A short fragment thought to correspond to the terminal unit on the GAS carbohydrate has been proposed for use in a vaccine [116]. Accordingly, short fragments are envisaged in the present invention. However, it is preferred to use saccharides of substantially full-length. The GAS carbohydrate typically has a molecular weight of about 10, in particular about 7.5-8.5 kDa. Molecular masses can be measured by HPLC, for example SEC-HPLC using a TSK Gel G3000SW column (Sigma) relative to pullulan standards, such as those available from Polymer Standard Service [117].

The saccharide may be chemically modified relative to the GAS carbohydrate as found in nature. For example, the saccharide may be de-N-acetylated (partially or fully), N-propionated (partially or fully), *etc.* The effect of de-acetylation *etc.*, for example on immunogenicity, can be assessed by routine assays.

**The conjugate**

The invention involves a conjugate comprising an antigen and a carrier molecule, wherein the carrier molecule comprises a spr0096 antigen and a spr2021 antigen.

The carrier molecule may be covalently conjugated to the antigen directly or via a linker. Any suitable conjugation reaction can be used, with any suitable linker where desired.

Attachment of the antigen to the carrier is preferably via a -NH₂ group *e.g.* in the side chain of a lysine residue in a carrier protein, or of an arginine residue. Where the antigen has a free aldehyde group, then this can react with an amine in the carrier to form a conjugate by reductive amination. Attachment to the carrier may also be via a -SH group *e.g.* in the side chain of a cysteine residue. Alternatively the antigen may be attached to the carrier via a linker molecule.

The antigen will typically be activated or functionalised prior to conjugation. Activation may involve, for example, cyanating reagents such as CDAP (*e.g.* 1-cyano-4-dimethylamino pyridinium tetrafluoroborate [118, 119, *etc.*]). Other suitable techniques use carbodiimides, hydrazides, active
esters, norborane, p-nitrobenzoic acid, N-hydroxysuccinimide, S-NHS, EDC, TSTU (see also the introduction to reference 7).

Direct linkages to the protein may comprise oxidation of the antigen followed by reductive amination with the protein, as described in, for example, references 120 and 121.

5 Linkages via a linker group may be made using any known procedure, for example, the procedures described in references 122 and 123. Typically, the linker is attached via the anomic carbon of a saccharide antigen. A preferred type of linkage is an adipic acid linker, which may be formed by coupling a free -NH₂ group (e.g. introduced to a saccharide by amination) with adipic acid (using, for example, diimidide activation), and then coupling a protein to the resulting antigen-adipic acid intermediate [5, 124, 125]. A similar preferred type of linkage is a glutaric acid linker, which may be formed by coupling a free -NH₂ group with glutaric acid in the same way. Adipic and glutaric acid linkers may also be formed by direct coupling to the antigen, i.e. without prior introduction of a free group, e.g. a free -NH₂ group, to the antigen, followed by coupling a protein to the resulting antigen-adipic/glutaric acid intermediate. Another preferred type of linkage is a carbonyl linker, which may be formed by reaction of a free hydroxyl group of a modified antigen with CDI [126, 127] followed by reaction with a protein to form a carbamate linkage. Other linkers include β-propionamido [128], nitrophenyl-ethylamine [129], haloacyl halides [130], glycosidic linkages [131], 6-aminoacaproic acid [132], N-succinimidyl-3-(2-pyridyldithio)-propionate (SPDP) [133], adipic acid dihydrazide ADH [134], C₄ to C₁₂ moieties [135], etc. Carbodiimide condensation can also be used [136].

20 A bifunctional linker may be used to provide a first group for coupling to an amine group in the antigen (e.g. introduced to the antigen by amination) and a second group for coupling to the carrier (typically for coupling to an amine in the carrier). Alternatively, the first group is capable of direct coupling to the antigen, i.e. without prior introduction of a group, e.g. an amine group, to the antigen.

25 In some embodiments, the first group in the bifunctional linker is thus able to react with an amine group (-NH₂) on the antigen. This reaction will typically involve an electrophilic substitution of the amine’s hydrogen. In other embodiments, the first group in the bifunctional linker is able to react directly with the antigen. In both sets of embodiments, the second group in the bifunctional linker is typically able to react with an amine group on the carrier. This reaction will again typically involve an electrophilic substitution of the amine.

30 Where the reactions with both the antigen and the carrier involve amines then it is preferred to use a bifunctional linker. For example, a homobifunctional linker of the formula X-L-X may be used, where: the two X groups are the same as each other and can react with the amines; and where L is a linking moiety in the linker. Similarly, a heterobifunctional linker of the formula X-L-X may be used, where: the two X groups are different and can react with the amines; and where L is a linking moiety in the linker. A preferred X group is N-oxyssuccinimide. L preferably has formula L'-L²-L', where L' is carbonyl. Preferred L² groups are straight chain alkyls with 1 to 10 carbon atoms (e.g. C₁, C₂, C₃, C₄, C₅, C₆, C₇, C₈, C₉, C₁₀) e.g. -(CH₂)ₙ- or -(CH₂)₁₀-.

35
Similarly, where the reaction with the antigen involves direct coupling and the reaction with the carrier involves an amine then it is also preferred to use a bifunctional linker. For example, a homobifunctional linker of the formula X-L-X may be used, where: the two X groups are the same as each other and can react with the antigen/amine; and where L is a linking moiety in the linker.

Similarly, a heterobifunctional linker of the formula X-L-X may be used, where: the two X groups are different and one can react with the antigen while the other can react with the amine; and where L is a linking moiety in the linker. A preferred X group is N-oxysuccinimide. L preferably has formula L'-L²-L', where L' is carbonyl. Preferred L² groups are straight chain alkyls with 1 to 10 carbon atoms (e.g. C₁, C₂, C₃, C₄, C₅, C₆, C₇, C₈, C₉, C₁₀) e.g. -(CH₂)₄- or -(CH₂)₃-. Other X groups for use in the bifunctional linkers described in the two preceding paragraphs are those which form esters when combined with HO-L-OH, such as norborane, p-nitrobenzoic acid, and sulfo-N-hydroxysuccinimide.

Further bifunctional linkers for use with the invention include acryloyl halides (e.g. chloride) and haloacetylhalides.

The linker will generally be added in molar excess to antigen during coupling to the antigen.

When the antigen has a single group that is linked to the carrier molecule (optionally via a linker), and the carrier has multiple groups that are linked to different antigen/linker molecules, the resultant conjugate may form a “star” structure. This structure comprises a central carrier molecule with multiple antigen molecules radiating from the carrier (optionally via linkers). When the antigen has more than one group that is linked to the carrier molecule (optionally via a linker), and the carrier has more than one group that is linked to different antigen/linker molecules, the resultant conjugate may form a “net” structure. This structure comprises a network of carrier molecules connected by antigen molecules (optionally via linkers).

Conjugates may have excess carrier (w/w) or excess antigen (w/w) e.g. in the ratio range of 1:5 to 5:1. Conjugates with excess carrier protein are typical e.g. in the range 0.2:1 to 0.9:1, or equal weights. The conjugate may include small amounts of free (i.e. unconjugated) carrier. When a given carrier protein is present in both free and conjugated form in a composition of the invention, the unconjugated form is preferably no more than 5% of the total amount of the carrier protein in the composition as a whole, and more preferably present at less than 2% (by weight).

When the conjugate is comprised within a pharmaceutical composition of the invention, the composition may also comprise free carrier protein as immunogen [137]. After conjugation, free and conjugated antigens can be separated. There are many suitable methods e.g. hydrophobic chromatography, tangential ultrafiltration, diafiltration, etc. [see also refs. 138, 139 etc.]. Tangential flow ultrafiltration is preferred.

A saccharide moiety in the conjugate is preferably a low molecular weight saccharide or an oligosaccharide, as defined above. Oligosaccharides will typically be sized prior to conjugation.
The conjugate is preferably soluble in water and/or in a physiological buffer.

**Production and conjugation of carrier molecules modified to incorporate non-natural amino acids**

Where one or more non-natural amino acid residues is to be incorporated into the carrier molecule, then this can be performed using standard procedures. One such method comprises the use of modified host cells in which the amino acyl tRNA synthetase for a specific codon has been engineered to conjugate the tRNA to a non-natural amino acid which is then incorporated into the carrier during translation [see ref. 140 for a review of such techniques]. Alternatively, some procedures exploit the fact that some non-natural amino acids are incorporated into proteins by the native cellular machinery when the natural cognate amino acid is not present. An example of this second type of procedure is observed in the incorporation of HAG. Here, in some cells, if the cell has low or no methionine, then the native cellular machinery will incorporate HAG in place of methionine in the initiation and elongation steps of protein synthesis. Many host cells used for protein expression are prototrophic for methionine, i.e. the cell can synthesise this amino acid *de novo*. By using cells that are methionine auxotrophs, therefore, it is possible to lower the levels of methionine to such a low level that HAG is incorporated into proteins in place of methionine. Example methionine auxotrophic host cells include *E. coli* strains B834 (DE3) (Merck) and T7 Express Crystal (NEB), although other suitable strains will be immediately apparent to the skilled person.

The conjugation technique/reaction used to conjugate the carrier molecule should be appropriate for the functional group in the non-natural amino acid. For example, where the non-natural amino acid is HAG, then thiyl-ene conjugation is used [see, e.g., ref. 141].

**Mixtures comprising the conjugates**

The conjugates of the invention may be mixed with further antigens. These further antigens may be other conjugates of the invention or they may be other antigens.

For example, mixtures of conjugates are envisaged. At least one of the conjugates in these mixtures is a conjugate of the invention, *i.e.* the carrier molecule comprises a spr0096 antigen and a spr2021 antigen. Typically, the other conjugate(s) in these mixtures will also be conjugates of the invention. However, when the other conjugate(s) are not conjugates of the invention, the carrier molecule may be any suitable carrier protein (as described below), typically the same carrier molecule in each conjugate.

For example, mixtures of conjugates from more than one serogroup of *N.meningitidis* are envisaged *e.g.* compositions comprising saccharides from serogroups A+C, A+W135, A+Y, C+W135, C+Y, W135+Y, A+C+W135, A+C+Y, C+W135+Y, A+C+W135+Y, *etc.* Typically, the mixture is a mixture of conjugates comprising saccharides from serogroups A, C, W135 and Y. At least one of the conjugates in these mixtures is a conjugate of the invention, *i.e.* the carrier molecule comprises a spr0096 antigen and a spr2021 antigen. Typically, the other conjugate(s) in these mixtures will also
be conjugates of the invention. However, when the other conjugate(s) are not conjugates of the invention, the carrier molecule may be any suitable carrier protein (as described below), typically the same carrier molecule in each conjugate.

Suitable carrier proteins are bacterial toxins, such as diphtheria or tetanus toxins, or toxoids or mutants thereof. The inventors have found that the CRM197 diphtheria toxin mutant [142] is particularly suitable. Other suitable carrier proteins include the N. meningitidis outer membrane protein complex [143], synthetic peptides [144,145], heat shock proteins [146,147], pertussis proteins [148,149], cytokines [150], lymphokines [150], hormones [150], growth factors [150], human serum albumin (typically recombinant), artificial proteins comprising multiple human CD4+ T cell epitopes from various pathogen-derived antigens [17] such as N19 [151], protein D from H. influenzae [152-154], pneumococcal surface protein PspA [155], pneumolysin [156] or its non-toxic derivatives [157], iron-uptake proteins [158], toxin A or B from C. difficile [159], a GBS protein [160], a GAS protein [161] etc.

A single carrier protein might carry more than one polysaccharide antigen [162,163]. To achieve this goal, different saccharides can be mixed prior to the conjugation process. Typically, however, there are separate conjugates for each saccharide, with the different saccharides being mixed after conjugation. The separate conjugates may be based on the same carrier, particularly the same carrier comprising a spr0096 antigen and a spr2021 antigen.

A mixture of the invention may for example be a mixture of separate conjugates for each saccharide from serogroups A, C, W135 and Y, wherein the serogroup A conjugate is a conjugate of the invention, i.e. the carrier molecule comprises a spr0096 antigen and a spr2021 antigen, and the serogroup C, W135 and Y conjugates are not conjugates of the invention. In this embodiment, the carrier molecule in the serogroup C, W135 and Y conjugates is typically CRM197.

Where a mixture comprises capsular saccharides from both serogroups A and C, it is preferred that the ratio (w/w) of MenA saccharide:MenC saccharide is greater than 1 (e.g. 2:1, 3:1, 4:1, 5:1, 10:1 or higher).

Where a mixture comprises capsular saccharides from serogroup Y and one or both of serogroups C and W135, it is preferred that the ratio (w/w) of MenY saccharide:MenW135 saccharide is greater than 1 (e.g. 2:1, 3:1, 4:1, 5:1, 10:1 or higher) and/or that the ratio (w/w) of MenY saccharide:MenC saccharide is less than 1 (e.g. 1:2, 1:3, 1:4, 1:5, or lower).

The mixtures may also comprise proteins. For example, the mixtures may include proteins from serogroup B of *N.meningitidis* [e.g. refs. 164 to 169] or OMV preparations [e.g. refs. 170 to 173 etc.].

The further antigen(s) may comprise antigens from non-*N.meningitidis* pathogens. Thus the compositions of the invention may further comprise one or more non-*N.meningitidis* antigens, including additional bacterial, viral or parasitic antigens. These may be selected from the following:

- a saccharide antigen from *Streptococcus pneumoniae* [e.g. refs. 174-176; chapters 22 & 23 of ref. 183].
- an antigen from hepatitis A virus, such as inactivated virus [e.g. 177, 178; chapter 15 of ref. 183].
- an antigen from hepatitis B virus, such as the surface and/or core antigens [e.g. 178, 179; chapter 16 of ref. 183].
- an antigen from hepatitis C virus [e.g. 180].
- an antigen from *Bordetella pertussis*, such as pertussis holotoxin (PT) and filamentous haemagglutinin (FHA) from *B.pertussis*, optionally also in combination with pertactin and/or agglutinogens 2 and 3 [e.g. refs. 181 & 182; chapter 21 of ref. 183].
- a diphtheria antigen, such as a diphtheria toxoid [e.g. chapter 13 of ref. 183].
- a tetanus antigen, such as a tetanus toxoid [e.g. chapter 27 of ref. 183].
- a saccharide antigen from *Haemophilus influenzae* B [e.g. chapter 14 of ref. 183]
- an antigen from *N.gonorrhoeae* [e.g., 164 to 167]
- an antigen from *Chlamydia pneumoniae* [e.g. 184, 185, 186, 187, 188, 189, 190].
- an antigen from *Chlamydia trachomatis* [e.g. 191].
- an antigen from *Porphyromonas gingivalis* [e.g. 192].
- polio antigen(s) [e.g. 193, 194; chapter 24 of ref. 183] such as IPV.
- rabies antigen(s) [e.g. 195] such as lyophilised inactivated virus [e.g.196, RabAvert™].
- measles, mumps and/or rubella antigens [e.g. chapters 19, 20 and 26 of ref. 183].
- influenza antigen(s) [e.g. chapters 17 & 18 of ref. 183], such as the haemagglutinin and/or neuraminidase surface proteins.
- an antigen from *Moraxella catarrhalis* [e.g. 197].
- an antigen from *Streptococcus pyogenes* (group A streptococcus) [e.g. 198, 199, 200].
- an antigen from *Streptococcus agalactiae* (group B streptococcus) [e.g.160, 201-203].
- an antigen from *S.epidermidis* [e.g. type I, II and/or III capsular polysaccharide obtainable from strains ATCC-31432, SE-360 and SE-10 as described in refs. 204, 205 and 206].

Where a saccharide or carbohydrate antigen is used, it is typically conjugated to a carrier in order to enhance immunogenicity. The carrier molecule may be a carrier of the invention, *i.e.* a carrier that comprises a spr0096 antigen and a spr2021 antigen. Alternatively, the carrier molecule may be any
suitable carrier protein, e.g. as described above. Conjugation of *H. influenzae* B, meningococcal and pneumococcal saccharide antigens is well known.

Toxic protein antigens may be detoxified where necessary (e.g. detoxification of pertussis toxin by chemical and/or genetic means [182]).

Where a diphtheria antigen is included in the composition it is typical also to include tetanus antigen and pertussis antigens. Similarly, where a tetanus antigen is included it is typical also to include diphtheria and pertussis antigens. Similarly, where a pertussis antigen is included it is typical also to include diphtheria and tetanus antigens.

Antigens may be adsorbed to an aluminium salt.

Antigens in the composition will typically be present at a concentration of at least 1µg/ml each. In general, the concentration of any given antigen will be sufficient to elicit an immune response against that antigen.

As an alternative to using proteins antigens in the composition of the invention, nucleic acid encoding the antigen may be used [e.g. refs. 207 to 215]. Protein components of the compositions of the invention may thus be replaced by nucleic acid (usually DNA e.g. in the form of a plasmid) that encodes the protein.

In practical terms, there may be an upper limit to the number of antigens included in compositions of the invention. The number of antigens (including conjugates of the invention) in a composition of the invention may be less than 20, less than 19, less than 18, less than 17, less than 16, less than 15, less than 14, less than 13, less than 12, less than 11, less than 10, less than 9, less than 8, less than 7, less than 6, less than 5, less than 4, or less than 3. The number of conjugates of the invention in a composition may be less than 6, less than 5, or less than 4.

**Pharmaceutical compositions comprising the conjugates**

The invention provides a pharmaceutical composition comprising (a) a conjugate of the invention, and (b) a pharmaceutically acceptable carrier. A thorough discussion of such carriers is available in reference 216.

Microbial infections affect various areas of the body and so the compositions of the invention may be prepared in various forms. For example, the compositions may be prepared as injectables, either as liquid solutions or suspensions. Solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection can also be prepared. The composition may be prepared for topical administration e.g. as an ointment, cream or powder. The composition be prepared for oral administration e.g. as a tablet or capsule, or as a syrup (optionally flavoured). The composition may be prepared for pulmonary administration e.g. as an inhaler, using a fine powder or a spray. The composition may be prepared as a suppository or pessary. The composition may be prepared for nasal, aural or ocular administration e.g. as drops, as a spray, or as a powder [e.g. 217]. The composition may be included in a mouthwash. The composition may be lyophilised.

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The pharmaceutical composition is preferably sterile. It is preferably pyrogen-free. It is preferably buffered e.g. at between pH 6 and pH 8, generally around pH 7.

The invention also provides a delivery device containing a pharmaceutical composition of the invention. The device may be, for example, a syringe or an inhaler.

Pharmaceutical compositions of the invention are preferably immunogenic compositions, in that they comprise an immunologically effective amount of an antigen. By ‘immunologically effective amount’, it is meant that the administration of that amount to an individual, either in a single dose or as part of a series, is effective for treatment or prevention. This amount varies depending upon the health and physical condition of the individual to be treated, age, the taxonomic group of individual to be treated (e.g. non-human primate, primate, etc.), the capacity of the individual's immune system to synthesise antibodies, the degree of protection desired, the formulation of the vaccine, the treating doctor's assessment of the medical situation, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials. Dosage treatment may be a single dose schedule or a multiple dose schedule (e.g. including booster doses). The composition may be administered in conjunction with other immunoregulatory agents.

Once formulated, the compositions of the invention can be administered directly to the subject. The subjects to be treated can be animals; in particular, human subjects can be treated.

Immunogenic compositions of the invention may be used therapeutically (i.e. to treat an existing infection) or prophylactically (i.e. to prevent future infection). Therapeutic immunisation is particularly useful for treating Candida infection in immunocompromised subjects.

An immunogenic composition may include a further adjuvant, which can function to enhance the immune responses (humoral and/or cellular) elicited in a patient who receives the composition. Adjuvants that can be used with the invention include, but are not limited to:

- A mineral-containing composition, including calcium salts and aluminum salts (or mixtures thereof). Calcium salts include calcium phosphate (e.g. the “CAP” particles disclosed in ref. 218). Aluminum salts include hydroxides, phosphates, sulfates, etc., with the salts taking any suitable form (e.g. gel, crystalline, amorphous, etc.). Adsorption to these salts is preferred.

The mineral containing compositions may also be formulated as a particle of metal salt [219]. The adjuvants known as aluminum hydroxide and aluminum phosphate may be used. These names are conventional, but are used for convenience only, as neither is a precise description of the actual chemical compound which is present (e.g. see chapter 9 of reference 302). The invention can use any of the “hydroxide” or “phosphate” adjuvants that are in general use as adjuvants. The adjuvants known as “aluminium hydroxide” are typically aluminium oxyhydroxide salts, which are usually at least partially crystalline. The adjuvants known as “aluminium phosphate” are typically aluminium hydroxyphosphates, often also containing a small amount of sulfate (i.e. aluminium hydroxyphosphate sulfate). They may be obtained by
precipitation, and the reaction conditions and concentrations during precipitation influence the degree of substitution of phosphate for hydroxyl in the salt. The invention can use a mixture of both an aluminium hydroxide and an aluminium phosphate. In this case there may be more aluminium phosphate than hydroxide e.g. a weight ratio of at least 2:1 e.g. ≥5:1, ≥6:1, ≥7:1, ≥8:1, ≥9:1, etc. The concentration of Al⁺⁺⁺ in a composition for administration to a patient is preferably less than 10mg/ml e.g. ≤5 mg/ml, ≤4 mg/ml, ≤3 mg/ml, ≤2 mg/ml, ≤1 mg/ml, etc. A preferred range is between 0.3 and 1mg/ml. A maximum of 0.85mg/dose is preferred.

- Saponins [chapter 22 of ref. 302], which are a heterologous group of sterol glycosides and triterpenoid glycosides that are found in the bark, leaves, stems, roots and even flowers of a wide range of plant species. Saponin from the bark of the Quillaja saponaria Molina tree have been widely studied as adjuvants. Saponin can also be commercially obtained from Smilax ornata (sarsaparilla), Gypsophilla paniculata (brides veil), and Saponaria officinalis (soap root). Saponin adjuvant formulations include purified formulations, such as QS21, as well as lipid formulations, such as ISCOMs. QS21 is marketed as Stimulon™. Saponin compositions have been purified using HPLC and RP-HPLC. Specific purified fractions using these techniques have been identified, including QS7, QS17, QS18, QS21, QHA, QHB and QHC. Preferably, the saponin is QS21. A method of production of QS21 is disclosed in ref. 220. Saponin formulations may also comprise a sterol, such as cholesterol [221]. Combinations of saponins and cholesterol can be used to form unique particles called immunostimulating complexes (ISCOMs) [chapter 23 of ref. 302]. ISCOMs typically also include a phospholipid such as phosphatidylethanolamine or phosphatidylcholine. Any known saponin can be used in ISCOMs. Preferably, the ISCOM includes one or more of Quila, QHA & QHC. ISCOMs are further described in refs. 221-223. Optionally, the ISCOMS may be devoid of additional detergent [224]. A review of the development of saponin based adjuvants can be found in refs. 225 & 226.

- Bacterial ADP-ribosylating toxins (e.g. the E.coli heat labile enterotoxin “LT”, cholera toxin “CT”, or pertussis toxin “PT”) and detoxified derivatives thereof, such as the mutant toxins known as LT-K63 and LT-R72 [227]. The use of detoxified ADP-ribosylating toxins as mucosal adjuvants is described in ref. 228 and as parenteral adjuvants in ref. 229.

- Bioadhesives and mucoadhesives, such as esterified hyaluronic acid microspheres [230] or chitosan and its derivatives [231].

- Microparticles (i.e. a particle of ~100nm to ~150μm in diameter, more preferably ~200nm to ~30μm in diameter, or ~500nm to ~10μm in diameter) formed from materials that are biodegradable and non-toxic (e.g. a poly(α-hydroxy acid), a polyhydroxybutyric acid, a polyorthocester, a polyanhydride, a polycaprolactone, etc.), with poly(lactide-co-glycolide) being preferred, optionally treated to have a negatively-charged surface (e.g. with SDS) or a positively-charged surface (e.g. with a cationic detergent, such as CTAB).
- Liposomes (Chapters 13 & 14 of ref. 302). Examples of liposome formulations suitable for use as adjuvants are described in refs. 232-234.

- Muramyl peptides, such as N-acetylmuramyl-L-threonyl-D-isoglutamine (“thr-MDP”), N-acetyl-normuramyl-L-alanyl-D-isoglutamine (nor-MDP), N-acetylglysaminyl-N-acetylmuramyl-L-Al-D-isoglu-L-Ala-dipalmitoxy propylamide (“DTP-DPP”, or “Theramide™”), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1′,2′dipalmitoylsn-glycero-3-hydroxyphosphoryloxy)-ethylamine (“MTP-PE”).

- A polyoxidonium polymer [235,236] or other N-oxidized polyethylene-piperazine derivative.

- Methyl inosine 5′-monophosphate (“MIMP”) [237].

- A polyhydroxlated pyrrolizidine compound [238], such as one having formula:

```
HO
N
CH2OH
```

where R is selected from the group comprising hydrogen, straight or branched, unsubstituted or substituted, saturated or unsaturated acyl, alkyl (e.g. cycloalkyl), alkenyl, alkynyl and aryl groups, or a pharmaceutically acceptable salt or derivative thereof. Examples include, but are not limited to: casuarine, casuarine-6-a-D-glucopyranose, 3-epi-casuarine, 7-epi-casuarine, 3,7-diepi-casuarine, etc.

- A CD1d ligand, such as an a-glycosylceramide [239-246] (e.g. a-galactosylceramide), phytosphingosine-containing a-glycosylceramides, OCH, KRN7000 [(2S,3S,4R)-1-O-(a-D-galactopyranosyl)-2-(N-hexacosanoylamino)-1,3,4-octadecanetriol], CRONY-101, 3′-O-sulfo-galactosylceramide, etc.

- A gamma inulin [247] or derivative thereof, such as algammulin.

- An oil-in-water emulsion. Various such emulsions are known, and they typically include at least one oil and at least one surfactant, with the oil(s) and surfactant(s) being biodegradable (metabolisable) and biocompatible. The oil droplets in the emulsion are generally less than 5μm in diameter, and may even have a sub-micron diameter, with these small sizes being achieved with a microfluidiser to provide stable emulsions. Droplets with a size less than 220nm are preferred as they can be subjected to filter sterilization.

- An immunostimulatory oligonucleotide, such as one containing a CpG motif (a dinucleotide sequence containing an unmethylated cytosine residue linked by a phosphate bond to a guanosine residue), or a Cpi motif (a dinucleotide sequence containing cytosine linked to inosine), or a double-stranded RNA, or an oligonucleotide containing a palindromic sequence, or an oligonucleotide containing a poly(dG) sequence. Immunostimulatory oligonucleotides can include nucleotide modifications/analogs such as phosphorothioate modifications and can be double-stranded or (except for RNA) single-stranded. References
248, 249 and 250 disclose possible analog substitutions e.g. replacement of guanosine with 2'-deoxy-7-deazaguanosine. The adjuvant effect of CpG oligonucleotides is further discussed in refs. 251-256. A CpG sequence may be directed to TLR9, such as the motif GTCGGTT or TTCGGT [257]. The CpG sequence may be specific for inducing a Th1 immune response, such as a CpG-A ODN (oligodeoxynucleotide), or it may be more specific for inducing a B cell response, such a CpG-B ODN. CpG-A and CpG-B ODNs are discussed in refs. 258-260. Preferably, the CpG is a CpG-A ODN. Preferably, the CpG oligonucleotide is constructed so that the 5' end is accessible for receptor recognition. Optionally, two CpG oligonucleotide sequences may be attached at their 3' ends to form “immunomers”. See, for example, references 257 & 261-263. A useful CpG adjuvant is CpG7909, also known as ProMune™ (Coley Pharmaceutical Group, Inc.). Another is CpG1826. As an alternative, or in addition, to using CpG sequences, TpG sequences can be used [264], and these oligonucleotides may be free from unmethylated CpG motifs. The immunostimulatory oligonucleotide may be pyrimidine-rich. For example, it may comprise more than one consecutive thymidine nucleotide (e.g. TTTT, as disclosed in ref. 264), and/or it may have a nucleotide composition with >25% thymidine (e.g. >35%, >40%, >50%, >60%, >80%, etc.). For example, it may comprise more than one consecutive cytosine nucleotide (e.g. CCCC, as disclosed in ref. 264), and/or it may have a nucleotide composition with >25% cytosine (e.g. >35%, >40%, >50%, >60%, >80%, etc.). These oligonucleotides may be free from unmethylated CpG motifs. Immunostimulatory oligonucleotides will typically comprise at least 20 nucleotides. They may comprise fewer than 100 nucleotides.

A particularly useful adjuvant based around immunostimulatory oligonucleotides is known as IC31™ [265]. Thus an adjuvant used with the invention may comprise a mixture of (i) an oligonucleotide (e.g. between 15-40 nucleotides) including at least one (and preferably multiple) Cpl motifs, and (ii) a polycationic polymer, such as an oligopeptide (e.g. between 5-20 amino acids) including at least one (and preferably multiple) Lys-Arg-Lys tripeptide sequence(s). The oligonucleotide may be a deoxynucleotide comprising 26-mer sequence 5'-((C)_15)-3' (SEQ ID NO: 1). The polycationic polymer may be a peptide comprising 11-mer amino acid sequence KKLKLKLKLKLK (SEQ ID NO: 2).

- 3-O-deacetylated monophosphoryl lipid A (‘3dMPL’, also known as ‘MPL™’) [266-269]. In aqueous conditions, 3dMPL can form micellar aggregates or particles with different sizes e.g. with a diameter <150nm or >500nm. Either or both of these can be used with the invention, and the better particles can be selected by routine assay. Smaller particles (e.g. small enough to give a clear aqueous suspension of 3dMPL) are preferred for use according to the invention because of their superior activity [270]. Preferred particles have a mean diameter less than 220nm, more preferably less than 200nm or less than 150nm or less than 120nm, and can even have a mean diameter less than 100nm. In most cases, however, the mean diameter will not be lower than 50nm.
- An imidazoquinoline compound, such as Imiquimod ("R-837") [271,272], Resiquimod ("R-848") [273], and their analogs; and salts thereof (e.g. the hydrochloride salts). Further details about immunostimulatory imidazoquinolines can be found in references 274 to 278.

- A thiosemicarbazone compound, such as those disclosed in reference 279. Methods of formulating, manufacturing, and screening for active compounds are also described in reference 279. The thiosemicarbazones are particularly effective in the stimulation of human peripheral blood mononuclear cells for the production of cytokines, such as TNF-α.

- A tryptanthrin compound, such as those disclosed in reference 280. Methods of formulating, manufacturing, and screening for active compounds are also described in reference 280. The thiosemicarbazones are particularly effective in the stimulation of human peripheral blood mononuclear cells for the production of cytokines, such as TNF-α.

- A nucleoside analog, such as: (a) Isatorabine (ANA-245; 7-thia-8-oxoguanosine):

![Nucleoside Analog](image)

and prodrugs thereof; (b) ANA975; (c) ANA-025-1; (d) ANA380; (e) the compounds disclosed in references 281 to 283 Loxoribine (7-allyl-8-oxoguanosine) [284].

- Compounds disclosed in reference 285, including: Acylopiperazine compounds, Indoleidione compounds, Tetrahydrindolizine (THIQ) compounds, Benzocyclodione compounds, Aminocarboxyvinyl compounds, Aminobenzimidazole quinolinone (ABIQ) compounds [286,287], Hydrazinamidine compounds, Benzophenone compounds, Isoxazolone compounds, Sterol compounds, Quinazolinone compounds, Pyrrole compounds [288], Anthraquinone compounds, Quinoxaline compounds, Triazine compounds, Pyrazinopyrimidine compounds, and Benzazole compounds [289].

- An aminoalkyl glucosaminide phosphate derivative, such as RC-529 [290,291].

- A phosphazene, such as poly[di(carboxylatophenoxy)phosphazene] ("PCPP") as described, for example, in references 292 and 293.

- A substituted urea or compound of formula I, II or III, or a salt thereof:
as defined in reference 294, such as ‘ER 803058’, ‘ER 803732’, ‘ER 804053’, ER 804058’,
‘ER 804059’, ‘ER 804442’, ‘ER 804680’, ‘ER 804764’, ER 803022 or ‘ER 804057’ e.g.:

- Derivatives of lipid A from *Escherichia coli* such as OM-174 (described in refs. 295 & 296).
- Compounds containing lipids linked to a phosphate-containing acyclic backbone, such as the
  TLR4 antagonist E5564 [297,298]:
These and other adjuvant-active substances are discussed in more detail in references 302 & 303.

Antigens and adjuvants in a composition will typically be in admixture.

Compositions may include two or more of said adjuvants. For example, they may advantageously include both an oil-in-water emulsion and 3dMPL, etc.

Specific oil-in-water emulsion adjuvants useful with the invention include, but are not limited to:

- A submicron emulsion of squalene, Tween 80, and Span 85. The composition of the emulsion by volume can be about 5% squalene, about 0.5% polysorbate 80 and about 0.5% Span 85. In weight terms, these ratios become 4.3% squalene, 0.5% polysorbate 80 and 0.48% Span 85. This adjuvant is known as ‘MF59’ [299-301], as described in more detail in Chapter 10 of ref. 302 and chapter 12 of ref. 303. The MF59 emulsion advantageously includes citrate ions e.g. 10mM sodium citrate buffer.

- An emulsion of squalene, a tocopherol, and Tween 80. The emulsion may include phosphate buffered saline. It may also include Span 85 (e.g. at 1%) and/or lecithin. These emulsions may have from 2 to 10% squalene, from 2 to 10% tocopherol and from 0.3 to 3% Tween 80, and the weight ratio of squalene:tocopherol is preferably ≤1 as this provides a more stable emulsion. Squalene and Tween 80 may be present volume ratio of about 5:2. One such emulsion can be made by dissolving Tween 80 in PBS to give a 2% solution, then mixing 90ml of this solution with a mixture of (5g of DL-α-tocopherol and 5ml squalene), then microfluidising the mixture. The resulting emulsion may have submicron oil droplets e.g. with an average diameter of between 100 and 250nm, preferably about 180nm.

- An emulsion of squalene, a tocopherol, and a Triton detergent (e.g. Triton X-100). The emulsion may also include a 3d-MPL (see below). The emulsion may contain a phosphate buffer.

- An emulsion comprising a polysorbate (e.g. polysorbate 80), a Triton detergent (e.g. Triton X-100) and a tocopherol (e.g. an α-tocopherol succinate). The emulsion may include these three components at a mass ratio of about 75:11:10 (e.g. 750μg/ml polysorbate 80, 110μg/ml Triton X-100 and 100μg/ml α-tocopherol succinate), and these concentrations should include any contribution of these components from antigens. The emulsion may also include squalene.
The emulsion may also include a 3d-MPL (see below). The aqueous phase may contain a phosphate buffer.

- An emulsion of squalane, polysorbate 80 and poloxamer 401 ("Pluronic™ L121"). The emulsion can be formulated in phosphate buffered saline, pH 7.4. This emulsion is a useful delivery vehicle for muramyl dipeptides, and has been used with threonyl-MDP in the "SAF-1" adjuvant [304] (0.05-1% Thr-MDP, 5% squalane, 2.5% Pluronic L121 and 0.2% polysorbate 80). It can also be used without the Thr-MDP, as in the "AF" adjuvant [305] (5% squalane, 1.25% Pluronic L121 and 0.2% polysorbate 80). Microfluidisation is preferred.

- An emulsion having from 0.5-50% of an oil, 0.1-10% of a phospholipid, and 0.05-5% of a non-ionic surfactant. As described in reference 306, preferred phospholipid components are phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidylglycerol, phosphatidic acid, sphingomyelin and cardiolipin. Submicron droplet sizes are advantageous.

- A submicron oil-in-water emulsion of a non-metabolisable oil (such as light mineral oil) and at least one surfactant (such as lecithin, Tween 80 or Span 80). Additives may be included, such as QuilA saponin, cholesterol, a saponin-lipophile conjugate (such as GPI-0100, described in reference 307, produced by addition of aliphatic amine to desacylsaponin via the carboxyl group of glucuronic acid), dimethyldioctadecylammonium bromide and/or N,N-dioctadecyl-N,N-bis (2-hydroxyethyl)propanediamine.

- An emulsion in which a saponin (e.g. QuilA or QS21) and a sterol (e.g. a cholesterol) are associated as helical micelles [308].

**Medical treatments and uses**

The invention also provides a conjugate of the invention, for use in medicine e.g. for use in raising an antibody response in a mammal.

The invention also provides a method for raising an immune response in a mammal, comprising administering a conjugate or pharmaceutical composition of the invention to the mammal.

The invention also provides the use of a conjugate of the invention in the manufacture of a medicament for preventing or treating a microbial infection in a mammal.

The immune response raised by these methods and uses will generally include an antibody response, preferably a protective antibody response. Methods for assessing antibody responses after antigen immunisation are well known in the art. The antibody response is preferably an IgA or IgG response. The immune response may be prophylactic and/or therapeutic. The mammal is preferably a human.

Efficacy of therapeutic treatment can be tested by monitoring microbial infection after administration of the composition of the invention. Efficacy of prophylactic treatment can be tested by monitoring immune responses against antigen (e.g. anti-antigen antibodies) after administration of the composition.
Compositions of the invention will generally be administered directly to a patient. Direct delivery may be accomplished by parenteral injection (e.g. subcutaneously, intraperitoneally, intravenously, intramuscularly, or to the interstitial space of a tissue), or by rectal, oral, vaginal, topical, transdermal, intradermal, ocular, nasal, aural, or pulmonary administration. Injection or intranasal administration is preferred.

The invention may be used to elicit systemic and/or mucosal immunity.

Vaccines prepared according to the invention may be used to treat both children and adults. Thus a subject may be less than 1 year old, 1-5 years old, 5-15 years old, 15-55 years old, or at least 55 years old. Preferred subjects for receiving the vaccines are the elderly (e.g. ≥50 years old, ≥60 years old, and preferably ≥65 years), or the young (e.g. ≤5 years old). The vaccines are not suitable solely for these groups, however, and may be used more generally in a population.

Treatment can be by a single dose schedule or a multiple dose schedule. Multiple doses may be used in a primary immunisation schedule and/or in a booster immunisation schedule. In a multiple dose schedule the various doses may be given by the same or different routes e.g. a parenteral prime and mucosal boost, a mucosal prime and parenteral boost, etc. Administration of more than one dose (typically two doses) is particularly useful in immunologically naïve patients. Multiple doses will typically be administered at least 1 week apart (e.g. about 2 weeks, about 3 weeks, about 4 weeks, about 6 weeks, about 8 weeks, about 10 weeks, about 12 weeks, about 16 weeks, etc.).

The uses and methods of the invention are particularly useful for treating/protection against infections caused by the organism from which the antigen is derived. Exemplary uses/methods are discussed below.

*N. meningitidis capsular saccharides*

The uses and methods may be for the prevention and/or treatment of a disease caused by *N. meningitidis*, e.g. meningitis, septicaemia, etc.

*Glucans*

Because glucans (and β-glucans in particular) are an essential and principal polysaccharide constituent of almost all pathogenic fungi, particularly those involved in infections in immunocompromised subjects, and also in bacterial pathogens and protozoa, anti-glucan immunity may have efficacy against a broad range of pathogens and diseases. For example, anti-glucan serum raised after immunisation with *S. cerevisiae* is cross-reactive with *C. albicans*. Broad spectrum immunity is particularly useful because, for these human infectious fungal agents, chemotherapy is scanty, antifungal drug resistance is emerging and the need for preventative and therapeutic vaccines is increasingly recognized.

The uses and methods of the invention are particularly useful for treating/protection against infections of: *Candida* species, such as *C. albicans*; *Cryptococcus* species, such as *C. neoformans*;
Enterococcus species, such as E. faecalis; Streptococcus species, such as S. pneumoniae, S. mutans, S. agalactiae and S. pyogenes; Leishmania species, such as L. major; Acanthamoeba species, such as A. castellani; Aspergillus species, such as A. fumigatus and A. flavus; Pneumocystis species, such as P. carinii; Mycobacterium species, such as M. tuberculosis; Pseudomonas species, such as P. aeruginosa; Staphylococcus species, such as S. aureus; Salmonella species, such as S. typhimurium; Coccioidoides species such as C. immitis; Trichophyton species such as T. verrucosum; Blastomyces species such as B. dermatitidis; Histoplasma species such as H. capsulatum; Paracoccidioides species such as P. brasiliensis; Pythium species such as P. insidiosum; and Escherichia species, such as E. coli.

The uses and methods are particularly useful for preventing/treating diseases including, but not limited to: candidiasis (including hepatosplenic candidiasis, invasive candidiasis, chronic mucocutaneous candidiasis and disseminated candidiasis); candidemia; aspergillosis, cryptococcosis, dermatomycoses, sporothrhyosis and other cutaneous mycoses, blastomycosis, histoplasmosis, coccidiomycosis, paracoccidiomycosis, pneumocystosis, thrush, tuberculosis, mycobacteriosis, respiratory infections, scarlet fever, pneumonia, impetigo, rheumatic fever, sepsis, septicemia, cutaneous and visceral leishmaniasis, corneal acanthamoebiasis, cystic fibrosis, typhoid fever, gastroenteritis and hemolytic-uremic syndrome. Anti-C. albicans activity is particularly useful for treating infections in AIDS patients.

Conjugates of the invention may be combined with non-glucan antigens into a single composition for simultaneous immunisation against multiple pathogens. As an alternative to making a combined vaccine, conjugates may be administered to patients at substantially the same time as (e.g. during the same medical consultation or visit to a healthcare professional or vaccination centre) other vaccines. Antigens for use in these combination vaccines or for concomitant administration include, for instance, immunogens from Streptococcus agalactiae, Staphylococcus aureus and/or Pseudomonas aeruginosa, hepatitis A virus, hepatitis B virus, Neisseria meningitidis (such as saccharides or conjugated saccharides, for serogroups A, C, W135 and/or Y), Streptococcus pneumoniae (such as saccharides or conjugated saccharides), etc.

Conjugates of the invention may be used in conjunction with anti-fungals, particularly where a patient is already infected. The anti-fungal offers an immediate therapeutic effect whereas the conjugate offers a longer-lasting effect. Suitable anti-fungals include, but are not limited to, azoles (e.g. fluconazole, itraconazole), polyenes (e.g. amphotericin B), flucytosine, and squalene epoxidase inhibitors (e.g. terbinafine) [see also ref. 309]. The anti-fungal and the conjugate may be administered separately or in combination. When administered separately, they will typically be administered within 7 days of each other. After the first administration of an conjugate, the anti-fungal may be administered more than once.

S. pneumoniae capsular saccharides

The uses and methods may be for the prevention and/or treatment of a disease caused by pneumococcus, e.g. meningitis, sepsis, pneumonia etc.
Definitions

The practice of the present invention will employ, unless otherwise indicated, conventional methods of chemistry, biochemistry, molecular biology, immunology and pharmacology, within the skill of the art. Such techniques are explained fully in the literature. See, e.g., references 216 and 310-316, etc.

“GI” numbering is used above. A GI number, or “GenInfo Identifier”, is a series of digits assigned consecutively to each sequence record processed by NCBI when sequences are added to its databases. The GI number bears no resemblance to the accession number of the sequence record. When a sequence is updated (e.g. for correction, or to add more annotation or information) then it receives a new GI number. Thus the sequence associated with a given GI number is never changed.

Where an antigen “domain” is omitted, this may involve omission of a signal peptide, of a cytoplasmic domain, of a transmembrane domain, of an extracellular domain, etc.

The term “comprising” encompasses “including” as well as “consisting” e.g. a composition “comprising” X may consist exclusively of X or may include something additional e.g. X + Y.

The term “about” in relation to a numerical value x is optional and means, for example, x±10%.

The word “substantially” does not exclude “completely” e.g. a composition which is “substantially free” from Y may be completely free from Y. Where necessary, the word “substantially” may be omitted from the definition of the invention.

The term “canonical” in relation to amino acids means that the amino acid is one of the twenty amino acids encoded by the universal genetic code, i.e. Alanine, Asparagine, Aspartic acid, Arginine, Cysteine, Glutamine, Glycine, Glutamic acid, Histidine, Isoleucine, Lysine, Leucine, Phenylalanine, Methionine, Serine, Proline, Tryptophan, Threonine, Tyrosine and Valine.

Unless specifically stated, a process comprising a step of mixing two or more components does not require any specific order of mixing. Thus components can be mixed in any order. Where there are three components then two components can be combined with each other, and then the combination may be combined with the third component, etc.

Where animal (and particularly bovine) materials are used in the culture of cells, they should be obtained from sources that are free from transmissible spongiform encephalopathies (TSEs), and in particular free from bovine spongiform encephalopathy (BSE). Overall, it is preferred to culture cells in the total absence of animal-derived materials.

Where a compound is administered to the body as part of a composition then that compound may alternatively be replaced by a suitable prodrug.

References to a percentage sequence identity between two amino acid sequences means that, when aligned, that percentage of amino acids are the same in comparing the two sequences. This alignment and the percent homology or sequence identity can be determined using software programs known in
the art, for example those described in section 7.7.18 of ref. 317. A preferred alignment is determined by the Smith-Waterman homology search algorithm using an affine gap search with a gap open penalty of 12 and a gap extension penalty of 2, BLOSUM matrix of 62. The Smith-Waterman homology search algorithm is disclosed in ref. 318.

5 MODES FOR CARRYING OUT THE INVENTION

A. Production and purification of conjugates

Laminarin, pneumococcal capsular saccharide from serotype 5 and meningococcal capsular oligosaccharides from serogroups A, C, W135 and Y were conjugated to various known and experimental carrier proteins and purified, as described below.

10 **Lam-96/2021:** Laminarin was conjugated to SEQ ID NO: 9 according to the method of ref. 319 in phosphate buffered saline using activated laminarin at a molar ratio of polysaccharide ester groups to protein of 30 and a protein concentration of 2-10 mg/ml. Conjugates were purified by Immobilized Metal ion Affinity Chromatography (IMAC), making use of the histidine tag on the carrier protein. The purification was performed with His MultiTrap HP plates™ (GE Healthcare), prepacked 96-well filter plates for small-scale purification of histone-tagged proteins, with the use of a vacuum source. The purified conjugates were characterized by SDS-Page; MicroBCA for protein content; and HPAEC-PAD for saccharide content. Two lots had the following properties:

<table>
<thead>
<tr>
<th>Lot</th>
<th>Saccharide (µg/ml)</th>
<th>Protein (µg/ml)</th>
<th>Saccharide:protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>“Old”</td>
<td>141.0</td>
<td>275.0</td>
<td>0.5</td>
</tr>
<tr>
<td>“New”</td>
<td>191.4</td>
<td>333.0</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Laminarin was conjugated to SEQ ID NO: 10 (Lam-2021/96) by the same method. For comparison, laminarin was similarly conjugated to the following carrier proteins: CRM197, spr0565B (SEQ ID NO: 11), spr1416 (SEQ ID NO: 12), spr1418 (SEQ ID NO: 13), spr2021 (SEQ ID NO: 3) and spr0096 (SEQ ID NO: 1).

**MenA-, C-, W- and Y-96/2021:** meningococcal capsular oligosaccharides from serogroups A, C, W135 and Y were conjugated to SEQ ID NO: 9 using the method described in reference 320. The conjugates were purified by IMAC as described above. The purified conjugates were characterized by SDS-Page, MicroBCA and HPAEC-PAD. Representative conjugates had the following properties:
<table>
<thead>
<tr>
<th>Conjugate</th>
<th>Saccharide (μg/ml)</th>
<th>Protein (μg/ml)</th>
<th>Saccharide:protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>MenA-96/2021</td>
<td>66</td>
<td>223</td>
<td>0.3</td>
</tr>
<tr>
<td>MenC-96/2021</td>
<td>71</td>
<td>240</td>
<td>0.3</td>
</tr>
<tr>
<td>MenW135-96/2021</td>
<td>91</td>
<td>129</td>
<td>0.7</td>
</tr>
<tr>
<td>MenY-96/2021</td>
<td>70</td>
<td>160</td>
<td>0.4</td>
</tr>
</tbody>
</table>

For comparison, these saccharides were similarly conjugated to CRM197 using the method described in reference 320. A MenA-CRM197 conjugate with a higher saccharide:protein (hereinafter, “high glycosylation”) was also made using this method. The meningococcal capsular oligosaccharide from serogroup C was also conjugated to spr1416 (SEQ ID NO: 12).

**Pneumo type 5-96/2021**: pneumococcal capsular saccharide from serotype 5 was sized in a Sephacryl S300 column and oxidised in a solution of NaPi 10mM, NaCl 500mM at pH 7.2 with NaIO4 (30% mol of mol PS repeating unit) at room temperature over night. The material was purified by dialysis with 6-8 kDa cut-off membrane against water. Conjugation was carried out by reductive amination using the oxidized saccharide in Na2B4O7 100mM, NaCl 100mM at pH 8.4 (5mg/ml) at a saccharide:protein ratio of 1:1 (w/w) and a protein:NaBH4CN ratio of 1:1 (w/w) at 37°C for 48 hours. The conjugates were purified by adding solid ammonium sulphate (500g/l) to the solution of crude conjugate, holding the mixture for 30 minutes at 0°C to allow the conjugate to precipitate and then centrifuging and dissolving the pellet in NaPi 10mM at pH 7.2.

The purified conjugates were characterized by SDS-Page, MicroBCA and HPAEC-PAD. Representative conjugates had the following properties:

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>Saccharide (μg/ml)</th>
<th>Protein (μg/ml)</th>
<th>Saccharide:protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pneumo type 5-96/2021</td>
<td>258</td>
<td>680</td>
<td>0.38</td>
</tr>
</tbody>
</table>

For comparison, this saccharide was similarly conjugated to CRM197.
**B. Immunogenicity of Lam-96/2021 compared to other laminarin-pneumococcal protein conjugates**

The immunogenicity of the Lam-96/2021 conjugate was compared to laminarin conjugated to other carrier proteins derived from pneumococcal protein antigens. Briefly, saccharide conjugates (at 5μg saccharide dose) with or without alum adjuvant were administered subcutaneously to Balb/C mice at days 1, 14 and 28. The mice were bled at day 42 and specific anti-laminarin antibodies measured by ELISA (using plates coated with unconjugated laminarin [321]).

Results are shown in Figure 2, with the vertical dotted lines denoting separate studies. In the first study, the Lam-96/2021 conjugate was more immunogenic than laminarin conjugates based on spr0565B, spr1416 and spr1418 pneumococcal protein antigens. The conjugate was also more immunogenic than a reference laminarin conjugate based on CRM197 (see Figure 3, in which the data from the first and third study in Figure 2 are combined). In the second study, the immunogenicity of a laminarin conjugate based on spr2021 alone was lower than this reference conjugate. In the third study, the Lam-96/2021 conjugate was more immunogenic than a laminarin conjugate based on spr0096 alone. The conjugate was also typically more immunogenic than the reference conjugates based on CRM197, both in the presence and absence of alum adjuvant.

**C. Immunogenicity of other saccharide conjugates based on 96/2021**

The immunogenicity of the MenA-, C-, W- and Y-96/2021 and pneumo type 5-96/2021 conjugates was tested using the same schedule as laminarin conjugates and compared to reference conjugates based on CRM197. Balb/C mice were immunized with 2μg MenA saccharide, 1 μg MenC saccharide, 1μg MenW saccharide and 1μg MenY saccharide per dose. Specific anti-polysaccharide antibodies were determined by ELISA assay (using plates coated with the native unconjugated polysaccharide).

Results are shown in Figure 4, with the vertical horizontal line denoting separate studies. In the first study, the pneumo type 5-96/2021 conjugate was shown to be immunogenic. The conjugate was more immunogenic than a reference conjugate based on CRM197 in the presence of alum (a similar result with alum was seen in the related immunogenicity study reported in Figure 5). In the second study, the MenA-, C-, W- and Y-96/2021 were shown to be immunogenic, with immunogenicity comparable to or better than that of the reference conjugates.

**D. Pneumo type 5-96/2021 conjugates provide protective immunity**

The pneumo type 5-96/2021 conjugate was compared to a reference pneumo type 5-CRM197 conjugate in a mouse model of protective immunity against pneumococceus serotype 5 infection. In this experiment, groups of ten mice were immunized intraperitoneally with the different immunogens (with or without alum as adjuvant) at days 0, 14 and 28. Two groups of mice immunized with PBS alone and PBS/alum, respectively, were used as negative controls. Two weeks after the last immunization, all groups were infected intraperitoneally with a lethal dose of a pneumococcal type 5 strain (STREP-5). The protective efficacy was assessed using measures of bacteremia reduction and
mortality. 24 hours post infection, the level of bacteremia was evaluated in each of the immunized groups and compared with that of the control groups. Mortality was followed for 10 days post infection.

Results are reported in Figure 6. Both conjugates conferred complete protection at doses of 1µg and 0.25µg in the presence of alum adjuvant. However, only the pneumo type 5-96/2021 conjugate was capable of conferring protection against mortality in the absence of adjuvant.

In a related study, these conjugates were compared with the saccharide or 96/2021 carrier protein alone and together. Both conjugates gave complete (pneumo type 5-96/2021 conjugate) or almost complete (pneumo type 5-CRM197) protection. In contrast, the saccharide and carrier (alone or together) were ineffective (Figure 7).

E. Immunogenicity of MenA-96/2021 conjugates

Post second immunization sera were pooled from mice administered with: a) MenA oligosaccharide conjugated to CRM197 or to 96/2021; b) a combination of MenA-, C-, W- and Y-96/2021 conjugates, with or without alum. These pools were tested by ELISA for anti serogroup A antibodies titer. Functionality of the antibodies elicited against the capsular polysaccharide was assessed in a serum bactericidal assay using rabbit complement (rSBA).

Figure 8 shows the results for the pooled sera. The MenA-96/2021 conjugates were more immunogenic than the MenA-CRM197 conjugates. The MenA-96/2021 conjugates remained immunogenic when combined with corresponding MenC, MenW135 and MenY conjugates. Similar observations were made when the post second immunization sera from individual mice were tested (Figure 9, SBA titres above bars).

In another study, post second immunization sera from individual mice administered with: a) MenA oligosaccharide conjugated to CRM197 or to 96/2021; b) combinations of the MenA-96/2021 conjugates with MenC-, W- and Y-CRM197 conjugates, with or without alum. These pools were tested by ELISA for anti serogroup A antibodies titer. Once again, the MenA-96/2021 conjugates were more immunogenic than the MenA-CRM197 conjugates (Figure 10, SBA titres above bars). The MenA-96/2021 conjugates remained immunogenic when they were combined with MenC-, W- and Y-CRM197 conjugates.

F. Immunogenicity of MenC-96/2021 conjugates

Post second immunization sera were pooled from mice administered with: a) MenC oligosaccharide conjugated to CRM197 or to 96/2021; b) a combination of MenA-, C-, W- and Y-96/2021 conjugates, with or without alum. These pools were tested by ELISA for anti serogroup C antibodies titer. Functionality of the antibodies elicited against the capsular polysaccharide was assessed in a serum bactericidal assay using rabbit complement (rSBA).
Figure 11 shows the results for the pooled sera, with SBA titres above the bars. The immunogenicity of the MenC-96/2021 conjugates seemed to be higher or comparable to that of the MenC-CRM197 conjugates. The MenC-96/2021 conjugates remained immunogenic when combined with corresponding MenC, MenW135 and MenY conjugates.

G. Immunogenicity of MenW-96/2021 conjugates

Post second immunization sera were pooled from mice administered with: a) MenW oligosaccharide conjugated to CRM197 or to 96/2021; b) a combination of MenA-, C-, W- and Y-96/2021 conjugates, with or without alum. These pools were tested by ELISA for anti-serogroup W antibodies titer.

Figure 12 shows the results for the pooled sera. The immunogenicity of the MenW-96/2021 conjugates seems to be higher (without alum) or comparable (with alum) to that of the MenW-CRM197 conjugates. The MenW-96/2021 conjugates remained immunogenic when combined with corresponding MenC, MenW135 and MenY conjugates.

H. Immunogenicity of MenY-96/2021 conjugates

Post second immunization sera were pooled from mice administered with: a) MenY oligosaccharide conjugated to CRM197 or to 96/2021; b) a combination of MenA-, C-, W- and Y-96/2021 conjugates, with or without alum. These pools were tested by ELISA for anti-serogroup Y antibodies titer.

As shown in Figure 13, the immunogenicity of the MenY-96/2021 conjugates seems to be comparable to that of the MenY-CRM197 conjugates, and remains immunogenic when combined with corresponding MenC, MenW135 and MenY conjugates.

I. T-cell response to MenC-96/2021 conjugates

The T-cell response to MenC oligosaccharide conjugated to CRM197, 96/2021 or spr1416 was compared. In this experiment, groups of eight CD1 mice were immunized subcutaneously with the different immunogens (1μg saccharide dose without alum) at days 0, 14 and 28. Two groups of mice immunized with PBS alone and the unconjugated MenC oligosaccharide (1μg saccharide dose), respectively, were used as negative controls. 21 days after the last immunization, serum from each mouse was tested for anti-polysaccharide antibodies by ELISA. Spleens were isolated from three mice in each group for analysis of T-cell cytokine profile by intracellular-staining multicolour FACS after in vitro antigen-specific restimulation.

ELISA results are reported in Figure 14. The immunogenicity of the MenC-96/2021 conjugates seemed to be higher or comparable to that of the MenC-CRM197 and MenC-spr1416 conjugates. CRM197 and 96/2021, but not spr1416, induced T cell responses (Figure 15).
J. Preparation of 96/2021 incorporating HAG

A nucleic acid encoding the 96/2021 hybrid of SEQ ID NO: 9 (in vector pET21+ or pET24+ (Merck)) was transformed into competent cells of the methionine auxotrophic E. coli strains B834(DE3) (Merck) and T7 express crystal (NEB) using standard procedures.

To produce proteins incorporating HAG, the cells were inoculated into defined media so that the precise concentration of methionine could be controlled. Either AB4 complex or M9 minimal medium which did not contain methionine was used as the base media, supplemented with antibiotics as appropriate for maintenance of the host and/or expression plasmid. The composition of each medium is as follows:

**AB4 without methionine:**

AB4base 2x: Alanine 1 g/L, Arginine 0.858 g/L, Asparagine 0.65 g/L, Aspartic acid 0.656 g/L, Cysteine 0.202 g/L, Glutamine 0.806 g/L, Glutamic acid 0.812 g/L, Glycine 1.036 g/L, Histidine 0.26 g/L, Isoleucine 0.594 g/L, Leucine 1.176 g/L, Lysine 0.806 g/L, Phenylalanine 0.554 g/L, Proline 0.476 g/L, Serine 4.108 g/L, Threonine 0.622 g/L, Tryptophan 0.222 g/L, Tyrosine 0.5 g/L, Valine 0.83 g/L, Adenine 1 g/L, Guanosine 0.858 g/L, Thymine 0.65 g/L, Uracil 0.656 g/L, Water 1000 ml.

Vit Mix 1000x: Riboflavin 1 g/L, Niacinamide 1 g/L, Piridoxinehydrochloride 1 g/L, Thiamine 1 g/L, Water 1000 ml.

Trace Elements 2000x: FeSO₄ . 7H₂O 5.6 g/L, MnCl₂ . 4H₂O 4.0 g/L, CoCl₂ . 6H₂O 5.6 g/L, CaCl₂ . 2H₂O 3.0 g/L, CuSO₄ 0.4 g/L, ZnSO₄ . 7H₂O 0.6 g/L, Water 1000 ml.

Glucose (25%): Glucose 250 g/L, Water 1000 ml.

Final AB4 media (1x): AB4base 2.0x 500 ml, Water 386.5 ml, PBS 20x 100 ml, Vit Mix 1 ml, Trace Elements 0.5 ml, Glucose (25%) 12 ml.

**M9:**

Solution I: Glucose 200 g/L, MgSO₄ . 7H₂O 4.9 g/L, CaCl₂ . 2H₂O 0.28 g/L, Water 1000 ml.

Solution II (pH 7.4): K₂HPO₄ 70 g/L, KH₂PO₄ 30 g/L, NaCl 5 g/L, (NH₄)₂SO₄ 6 g/L, Water 1000 ml.

To make the final media, solutions I/II were mixed in the ratio 85:5:10 (H₂O:Solution I:Solution II).

The B834(DE3) or T7 express crystal strain carrying a 96/2021 expression vector was inoculated into either AB4 or M9 base media supplemented with methionine to 0.176 g/L. Cells were grown at 25°C with shaking at 180 rpm until an OD₆₀₀ of 1.6 was reached, as measured by a DU530 spectrophotometer (Beckman). At this point the cells were pelleted by centrifugation at 10,000g for 30 minutes at 4°C and washed twice in fresh base medium without any methionine, in order to remove methionine from the cell pellet. Following this, the pellet was resuspended and inoculated.
into a fresh culture of base medium, this time supplemented with HAG to the same concentration as the methionine was present in the initial growth stage (no methionine was added to this medium). The culture was supplemented with IPTG to 1 mM in order to induce expression of the 96/2021 hybrid protein and incubated for a further 3-6 hours at 25°C with shaking at 180 rpm.

The resulting cells were lysed and the protein purified using an IMAC column, with any residual imidazole removed by dialysis. The mass of the purified protein was then measured by mass spectrometry (see Figure 16), and confirmed to have a mass approximately corresponding to the predicted weight of the protein of SEQ ID NO: 20 (i.e. SEQ ID NO: 9 in which each methionine has been substituted from L-homoallylglycine). This indicated that expression of the protein in the presence of HAG and the absence of methionine caused HAG to be substituted in place of methionine during expression of 96/2021.

**Immunisation study (1)**

*General assay protocol:* Balb/c mice were immunized by subcutaneous injection according to the schedule described below. The injection volume was 200 μl and the injection contained alum phosphate adjuvant.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mice per group</th>
<th>Immunogen</th>
<th>Antigen dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1-8</td>
<td>PBS</td>
<td>1 μg</td>
</tr>
<tr>
<td>2</td>
<td>9-16</td>
<td>MenA-CRM197</td>
<td>1 μg</td>
</tr>
<tr>
<td>3</td>
<td>17-24</td>
<td>MenA-CRM197 (high glycosylation)</td>
<td>1 μg</td>
</tr>
<tr>
<td>4</td>
<td>25-32</td>
<td>MenA-96/2021</td>
<td>1 μg</td>
</tr>
<tr>
<td>5</td>
<td>33-40</td>
<td>MenA-CRM197 + MenCWY</td>
<td>2 μg + 1,1,1, 1 μg</td>
</tr>
<tr>
<td>6</td>
<td>41-48</td>
<td>MenA-CRM197 (high glycosylation) + MenCWY</td>
<td>2 μg + 1,1,1, 1 μg</td>
</tr>
<tr>
<td>7</td>
<td>49-56</td>
<td>MenA-96/2021 + MenCWY</td>
<td>2 μg + 1,1,1, 1 μg</td>
</tr>
</tbody>
</table>

MenCWY = combination of MenC-, W- and Y-CRM197 prepared according to ref. 320.

The conjugates had the following properties:
<table>
<thead>
<tr>
<th>Sample</th>
<th>Saccharide (µg/mL)</th>
<th>Protein (µg/mL)</th>
<th>Sacc/Protein (w/w)</th>
<th>Sacc/Protein (mol/mol)</th>
<th>Free Sacc %</th>
<th>Endotoxin (EU/µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MenA-CRM197</td>
<td>1901.6</td>
<td>4523.7</td>
<td>0.42</td>
<td>5.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MenA-CRM197 (high glycosylation)</td>
<td>538.0</td>
<td>711.3</td>
<td>0.76</td>
<td>9.8</td>
<td>&lt;16.1</td>
<td>0.03</td>
</tr>
<tr>
<td>MenA-96/2021</td>
<td>110.4</td>
<td>405.0</td>
<td>0.27</td>
<td>3.7</td>
<td>10</td>
<td>0.81</td>
</tr>
</tbody>
</table>

The post third immunisation IgG antibody titre against serogroup A capsular polysaccharide and serum bactericidal antibody titre against serogroup A strain F8238 are shown in Figure 17. The serogroup A conjugates were immunogenic and induced bactericidal antibodies. Responses were slightly reduced when either of the CRM197 conjugates were combined with other CRM197 conjugates derived from serogroups C, W135 and Y, but still well above the control. In contrast, little or no reduction was seen when the MenA-96/2021 conjugate was combined with these conjugates. Accordingly, the use of 96/2021 as carrier may help to reduce any immune interference between the serogroup A conjugate and these conjugates.

The post third immunisation IgG antibody titre against serogroups C, W135 and Y capsular polysaccharide and serum bactericidal antibody titre against certain strains from these serogroups are shown in Figure 18. The use of 96/2021 as carrier for serogroup A did not affect the immune responses to these other polysaccharides.

It will be understood that the invention has been described by way of example only and modifications may be made whilst remaining within the scope and spirit of the invention.
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[122] US patent 4,882,317
[123] US patent 4,695,624
[128] WO00/10599
[131] US patents 4,673,574; 4,761,283; 4,808,700.
[133] US patent 5,204,098
[134] US patent 4,965,338
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[139] WO00/38711
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[158] WO01/72337
[159] WO00/61761.
[161] WO02/34771.
[167] WO00/22430.
[170] WO01/52885.
[184] WO02/02606.
[189] WO00/27994.
[190] WO00/37494.
[198] WO02/34771.
[201] WO03/093306.
[205] US4197290
[219] WO00/23105.
[221] WO96/33739.
[223] WO96/11711.
[224] WO00/07621.
[228] WO95/17211.
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[233] US 5,916,588
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[263] WO03/035836.
[264] WO01/22972.
[277] US patents 4689338, 4929624, 5238944, 5266575, 5268376, 5346905, 5352784, 5389640, 5395937, 5482936, 5494916, 5525612, 6083505, 6440992, 6627640, 6656938, 6660735, 6660747, 6664260, 6664264, 6664265, 6667312, 6670372, 6677347, 6677348, 6677349, 6683088, 6703402, 6743920, 6800624, 6809203, 6888000 and 6924293.
[286] US 6,605,617.
[294] WO03/011223.
[307] US patent 6,080,725.
[316] PCR (Introduction to Biotechniques Series), 2nd ed. (Newton & Graham eds., 1997, Springer Verlag)
[320] WO03/007985
The conjugate according to claim 1, wherein the spr0096 antigen comprises an amino acid sequence having 50% or more identity to SEQ ID NO: 1 or SEQ ID NO: 2.

3. The conjugate according to claim 1 or claim 2, wherein the spr2021 antigen comprises an amino acid sequence having 50% or more identity to SEQ ID NO: 3.

4. The conjugate according to any of the preceding claims, wherein the carrier molecule comprises the spr0096 antigen and the spr2021 antigen as a single polypeptide chain.

5. The conjugate according to claim 4, wherein the polypeptide chain is of the formula NH$_2$-A-{$\cdot$-X-L-$\cdot$}-n-B-COOH, wherein: A is an optional N-terminal amino acid sequence; B is an optional C-terminal amino acid sequence; n is an integer of 2 or more (e.g. 2, 3, 4, 5, 6, etc.); each X is an amino acid sequence of an spr0096 antigen or an spr2021 antigen, wherein at least one X is an spr0096 antigen and at least one X is an spr2021 antigen; and L is an optional linker amino acid sequence.

6. The conjugate according to claim 5, wherein n is 2.

7. The conjugate according to claim 6, wherein X$_1$ is an spr0096 antigen and X$_2$ is an spr2021 antigen.

8. The conjugate according to claim 7, wherein the polypeptide chain comprises an amino acid sequence having 50% or more identity (e.g. 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 9, particularly an amino acid sequence of SEQ ID NO: 9.

9. The conjugate according to any of the preceding claims, wherein the antigen is a saccharide.

10. The conjugate according to claim 9, wherein the saccharide is a capsular saccharide from N.meningitidis, a glucan or a capsular saccharide from S.pneumoniae.

11. The conjugate according to claim 10, wherein the saccharide is a capsular saccharide from N.meningitidis serogroup A, C, W135 or Y

12. The conjugate according to any of claims 1-8, wherein the antigen is a hapten.

13. The conjugate according to claim 12, wherein the hapten is an opiate, marijuana, amphetamine, cocaine, barbituate, glutethimide, methyprylon, chloral hydrate, methaqualone, benzodiazepine, LSD, nicotine, anticholinergic drug, antipsychotic drug, tryptamine, other psychomimetic drug,
sedative, phencyclidine, psilocybine, volatile nitrite, and other drug inducing physical and/or psychological dependence.

14. A conjugate according to any of the preceding claims for use in medicine.

15. A pharmaceutical composition comprising a conjugate according to any of the preceding claims in combination with a pharmaceutically acceptable carrier.

16. A pharmaceutical composition according to claim 15 comprising a mixture of conjugates comprising saccharides from serogroups A, C, W135 and Y.

17. The pharmaceutical composition according to claim 16, wherein the conjugates comprising saccharides from serogroups A, C, W135 and Y are separate conjugates for each saccharide.

18. The pharmaceutical composition according to claim 17, wherein the conjugates comprising saccharides from serogroups A, C, W135 and Y are based on the same carrier.

19. The pharmaceutical composition according to claim 18, wherein the same carrier is a carrier molecule as defined in any of claims 1-8.

20. A method for raising an immune response in a mammal, comprising administering a conjugate according to any of claims 1-14 or a pharmaceutical composition according to any of claims 15-19 to the mammal.
<table>
<thead>
<tr>
<th>Polysaccharide</th>
<th>Repeat unit</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Haemophilus influenzae</em> Type b ('PRP')</td>
<td>$\rightarrow 3)-(\beta)-D-Rib-(1\rightarrow 1)-D-Ribitol-(5\rightarrow OPO_3\rightarrow)$</td>
</tr>
<tr>
<td><em>Neisseria meningitides</em></td>
<td></td>
</tr>
<tr>
<td>Group A</td>
<td>$\rightarrow 6)-(\alpha)-D-ManpNAc(3OAc)-(1\rightarrow OPO_3\rightarrow)$</td>
</tr>
<tr>
<td>Group C</td>
<td>$\rightarrow 9)-(\alpha)-D-Neu5Ac(7/8OAc)-(2\rightarrow)$</td>
</tr>
<tr>
<td>Group W135</td>
<td>$\rightarrow 6)-(\alpha)-D-Galp-(1\rightarrow 4)-(\alpha)-D-Neu5Ac(9OAc)-(2\rightarrow)$</td>
</tr>
<tr>
<td>Group Y</td>
<td>$\rightarrow 6)-(\alpha)-D-Glcp-(1\rightarrow 4)-(\alpha)-D-Neu5Ac(9OAc)-(2\rightarrow)$</td>
</tr>
<tr>
<td><em>Salmonella enterica</em> Typhi Vi</td>
<td>$\rightarrow )-(\alpha)-D-GalpNAcA(3OAc)-(1\rightarrow)$</td>
</tr>
<tr>
<td><em>Streptococcus pneumoniae</em></td>
<td></td>
</tr>
<tr>
<td>Type 1</td>
<td>$\rightarrow 3)-(\beta)-AAT-(\alpha)-Galp-(1\rightarrow 4)-(\alpha)-D-GalpA(2/3OAc)-(1\rightarrow 3)-(\alpha)-D-GalpA-(1\rightarrow)$</td>
</tr>
<tr>
<td>Type 2</td>
<td>$\rightarrow 4)-(\beta)-D-Glcp-(1\rightarrow 3)-[(\alpha)-D-GlcpA-(1\rightarrow 6)-(\alpha)-D-Glcp-(1\rightarrow 2)]-(\alpha)-L-Rhap-(1\rightarrow 3)-(\alpha)-L-Rhap-(1\rightarrow)$</td>
</tr>
<tr>
<td>Type 3</td>
<td>$\rightarrow 3)-(\beta)-D-GlcA-(1\rightarrow 4)-(\beta)-D-Glcp-(1\rightarrow)$</td>
</tr>
<tr>
<td>Type 4</td>
<td>$\rightarrow 3)-(\beta)-D-ManpNAc-(1\rightarrow 3)-(\alpha)-L-FucpNAc-(1\rightarrow 3)-(\alpha)-D-GalpNAc-(1\rightarrow 4)-(\alpha)-D-Galp2,3(S)Py-(1\rightarrow)$</td>
</tr>
<tr>
<td>Type 5</td>
<td>$\rightarrow 4)-(\beta)-D-Glcp-(1\rightarrow 4)-[(\alpha)-L-PnepNAc-(1\rightarrow 2)-(\beta)-D-GlcpA-(1\rightarrow 3)]-(\alpha)-L-FucpNAc-(1\rightarrow 3)-(\beta)-D- SgpP-(1\rightarrow)$</td>
</tr>
<tr>
<td>Type 6B</td>
<td>(\rightarrow 2\cdot\alpha\cdot\text{D-Galp} (1\rightarrow 3)\cdot\alpha\cdot\text{D-GlcP} (1\rightarrow 3)\cdot\alpha\cdot\text{L-Rhap} (1\rightarrow 4)\cdot\text{D-Rib-ol} (5\rightarrow P))</td>
</tr>
<tr>
<td>Type 9N</td>
<td>(\rightarrow 4\cdot\alpha\cdot\text{D-GlcP A} (1\rightarrow 3)\cdot\alpha\cdot\text{D-GlcP} (1\rightarrow 3)\cdot\beta\cdot\text{D-ManpNAc} (1\rightarrow 4)\cdot\beta\cdot\text{D-GlcP} (1\rightarrow 4)\cdot\alpha\cdot\text{D-GlcP NAc} (1\rightarrow))</td>
</tr>
<tr>
<td>Type 14</td>
<td>(\rightarrow 4\cdot\beta\cdot\text{D-GlcP} (1\rightarrow 6)\cdot[\beta\cdot\text{D-Galp} (1\rightarrow 4)]\cdot\beta\cdot\text{D-GlcP NAc} (1\rightarrow 3)\cdot\beta\cdot\text{D-Galp} (1\rightarrow))</td>
</tr>
<tr>
<td>Type 18C</td>
<td>(\rightarrow 4\cdot\beta\cdot\text{D-GlcP} (1\rightarrow 4)\cdot[\alpha\cdot\text{D-GlcP} (6\text{OAc}) (1\rightarrow 2)]\cdot[\text{Gro} (1\rightarrow P) (3)]\cdot\beta\cdot\text{D-Galp} (1\rightarrow 4)\cdot\alpha\cdot\text{D-GlcP} (1\rightarrow 3)\cdot\beta\cdot\text{L-Rhap} (1\rightarrow))</td>
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<tr>
<td>Type 19A</td>
<td>(\rightarrow 4\cdot\beta\cdot\text{D-ManpNAc} (1\rightarrow 4)\cdot\alpha\cdot\text{D-GlcP} (1\rightarrow 3)\cdot\alpha\cdot\text{L-Rhap} (1\rightarrow P))</td>
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<tr>
<td>Type 19F</td>
<td>(\rightarrow 4\cdot\beta\cdot\text{D-ManpNAc} (1\rightarrow 4)\cdot\alpha\cdot\text{D-GlcP} (1\rightarrow 2)\cdot\alpha\cdot\text{L-Rhap} (1\rightarrow P))</td>
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<tr>
<td>Type 23F</td>
<td>(\rightarrow 4\cdot\beta\cdot\text{D-GlcP} (1\rightarrow 4)\cdot[\alpha\cdot\text{L-Rhap} (1\rightarrow 2)]\cdot[\text{Gro} (2\rightarrow P) (3)]\cdot\beta\cdot\text{D-Galp} (1\rightarrow 4)\cdot\beta\cdot\text{L-Rhap} (1\rightarrow))</td>
</tr>
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**Staphylococcus aureus**

| Type 5 | \(\rightarrow 4\cdot\beta\cdot\text{D-ManNAc A} (3\text{OAc}) (1\rightarrow 4)\cdot\alpha\cdot\text{L-FucNAc} (1\rightarrow 3)\cdot\beta\cdot\text{D-FucNAc} (1\rightarrow)\) |
| Type 8 | \(\rightarrow 3\cdot\beta\cdot\text{D-ManNAc A} (4\text{OAc}) (1\rightarrow 3)\cdot\alpha\cdot\text{L-FucNAc} (1\rightarrow 3)\cdot\beta\cdot\text{D-FucNAc} (1\rightarrow)\) |

AAT is 2-acetamido-4-amino-2,4,6-trideoxygalactose, Gro is glycerol, Pne is 2-acetamido-2,6-dideoxytalose, and P is phosphate in a phosphodiester linkage.

**FIG. 1 (contd)**
FIG. 5

- [Diagram]

- [Legend]

- [Y-axis labels: 10, 100, 1000, 10000, 100000]

- [X-axis labels: Pn typ5-CRM, Pn typ5-96/2021, PBS]
**FIG. 7(b)**

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<td>1 - 2.65 µg PS5-96/2021</td>
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<td>1 + 2.65 µg PS5 + 96/2021</td>
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<td>1 µg PS5-CRM</td>
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**INTERNATIONAL SEARCH REPORT**

**A. CLASIFICATION OF SUBJECT MATTER:**

**ADD.**

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)
A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, EMBASE, BIOSIS

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

<table>
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<th>Relevant to claim No.</th>
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<td>1-8,14, 15,20</td>
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* Further documents are listed in the continuation of Box C.  
X See patent family annex.

**Date of the actual completion of the international search**
31 January 2013

**Date of mailing of the international search report**
07/02/2013

**Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentliaan 2 NL - 2280 HV Rijswijk Tel: (+31-70) 340-0340, Fax: (+31-70) 340-3016**

Authorized officer
Monami, Amélie
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Form PCT/ISA/210 (continuation of second sheet) [April 2005]
# INTERNATIONAL SEARCH REPORT

Information on patent family members

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