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(54) Title: LYOPHILISED ANTIGEN COMPOSITION

(57) Abstract: The present invention provides lyophilised compositions comprising an antigen and a Toll- like receptor (TLR) 9 agonist. Such compositions may be reconstituted into immunogenic compositions for use in vaccination with a carrier selected from the group of particulate carriers consisting of liposomes, mineral salts, emulsions, polymers and ISCOMs. Methods of making immunogenic compositions from the lyophilised compositions of the invention and use of the same in immunisation are also herein provided.

# Lyophilised Antigen Composition

## Technical Field

The present invention relates to improved antigenic compositions and methods of using the same to make immunogenic compositions. In particular the present invention relates to lyophilised compositions comprising an antigen and a Toll-like receptor (TLR) 9 agonist. Such compositions may be reconstituted into immunogenic compositions for use in vaccination with a carrier selected from the group of particulate carriers consisting of liposomes, mineral salts, emulsions, polymers and ISCOMs. Methods of making immunogenic compositions from the lyophilised compositions of the invention and use of the same in immunisation are also part of the present invention.

## Background to the invention

Adjuvants are sometimes used to improve the immune response raised to any given antigen. However the inclusion of adjuvants into a vaccine or immunogenic composition increases the complexity of preparation of the components as well as the complexity of distribution and formulation of the vaccine composition. The preparation of each of the adjuvant components as well as the antigenic component must be considered by formulators. This is particularly true because for example the pH of adjuvant components in solution may be very different from the optimal pH for a given antigen and these differences need to be carefully controlled and managed to prevent, for example precipation or loss of desirable properties of the components. The pH of the antigen in water for injection may, for example be about pH7 or slightly higher and when the adjuvant is added the pH may be as low as pH6.3. The antigen may, for example not be stable when stored for prolonged periods at this pH.

The components must then be formulated and distributed in a form that is as stable as possible because pharmaceutical products for human use must be well characterized, stable and safe before they can be approved for marketing. For this reason long term stability studies must be performed on the final formulation to ensure that it meets the relevant criteria. The information generated in such long term studies is used to support submission to regulatory authorities such as the FDA (Federal Drugs Authority – the body responsible for approving medicines in the USA) to show the product is suitable for use in humans.

Freeze-drying or lyophilisation, is used generally to increase the stability and hence storage life of material including pharmaceutical materials such as an antigen used in vaccines.

Often lyophilised antigenic compositions are provided to health care professions for reconstitution with diluent (for example water for injection [WFI] or in some instances a liquid adjuvant formulation) shortly before administration to the patient. In this way the

period of time that the various components of the final vaccine are maintained in close proximity is minimised.

Many factors must be considered when antigens are lyophilised to form lyo cakes (the dry product from lyophilisation). For example, the antigenicity/immunogenicity of the antigen should be maintained in lyophilised form. The antigen must not aggregate or degrade whilst in lyophilised form. The lyo cake must be well formed and not collapse. Finally, the antigen must of course be in a form which dissolves rapidly when reconstituted. Where the solution for reconstitution is not simply WFI, for example when the antigen is reconstituted with liquid adjuvant, then the impact of the components of the solution on the properties of the reconstituted product needs to be considered.

As mentioned adjuvants have been used for many years to improve the immune response to the antigenic component of a vaccine. A particularly potent adjuvant combination is one comprising 3Deacylated-Monophosphoryl Lipid A (3D-MPL) and a saponin, particularly QS21, a purified fraction of saponin extracted from the bark of *Quillaja saponaria Monara*. This combination can be provided, for example as an oil in water emulsion, liposomal formulation or the like.

In previous clinical trials trials with antigens, for example with malaria antigens such as RTS,S the lyophilized antigen is provided and a separate vial of liquid adjuvant, for example an oil in water formulation of MPL and QS21 or a liposomal formulation of MPL and QS21 for reconstituting the antigen is also provided. The individual components are combined to form the final vaccine composition shortly before administration.

Certain immunostimulatory oligonucleotides containing unmethylated CpG dinucleotides ("CpG") are TLR9 ligands and have been identified as being adjuvants when administered by both systemic and mucosal routes (WO 96/02555, EP 468520, Davis *et al., J.Immunol*, 1998, 160(2):870-876; McCluskie and Davis, *J.Immunol.*, 1998, 161(9):4463-6). CpG is an abbreviation for cytosine-guanosine dinucleotide motifs present in DNA. Historically, it was observed that the DNA fraction of BCG could exert an anti-tumour effect. In further studies, synthetic oligonucleotides derived from BCG gene sequences were shown to be capable of inducing immunostimulatory effects (both in vitro and in vivo). The authors of these studies concluded that certain palindromic sequences, including a central CG motif, carried this activity. The central role of the CG motif in immunostimulation was later elucidated in a publication by Krieg, Nature 374, p546 1995. Detailed analysis has shown that the CG motif has to be in a certain sequence context, and that such sequences are common in bacterial DNA but are rare in vertebrate DNA. The immunostimulatory sequence is often: Purine, Purine, C, G, pyrimidine, pyrimidine; wherein the dinucleotide CG motif is not methylated, but other unmethylated CpG sequences are known to be immunostimulatory and may be used in the present invention.

It has also been shown that an immunostimulatory oligonucleotide can retain immunological activity when the Guanosine is mutated to a 7-deazaguanosine motif (*WO 03057822*).

These immunostimulatory oligonucleotides are thought to have an acidic pH in solution, for example below pH 7, such as 6.3, 6.1 or lower. This may make them difficult to incorporate in liquid vaccine formulations because they are dissimilar to other components in the formulations. As discussed this may cause precipitation and/or long term stability problems.

It is thought that these immunostimulatory oligonucleotides are likely to be very effective adjuvants, particularly when used in combination with exisiting adjuvant combinations such as 3D-MPL and QS21. It is expected that such adjuvants will be employed in diseases that have so far been difficult to provide effective vaccines for, such as HIV, cancer and possibly malaria.

There are a number of different ways in which adjuvants can be included in vaccines, but they must be included in a way which does not affect the stability either of themselves or the antigenic composition and also in a way which will not place an undue burden on the healthcare professional reconstituting the vaccine. The simplest way to achieve this would be to put additional components into additional vials such that they would be kept separate until just before reconstitution, thereby minimising the time during which the components could affect each other. This means the antigen and the immunostimulatory oligonucleotide would each be provided in a separate vials. Then if further adjuvant components such as MPL and QS21 are employed these can be provided as a liquid mixture in a third vial. However, an increasing number of components in an increasing number of vials leads to increased costs, waste and importantly to an increase in the possibility of mistakes during constitution.

**Summary of the Invention**

The present inventors have found that when a TLR9 ligand such as a CpG immunostimulatory oligonucleotide is to be part of an immunogenic composition as an adjuvant, said TLR9 ligand may be lyophilised together with the antigen such that there is provided a single vial containing antigen and TLR9 ligand adjuvant together in one lyo cake.

The present invention therefore provides a lyophilised composition comprising an antigen and a TLR9 agonist. Said TLR9 agonist in one embodiment is an immunostimulatory oligonucleotide, possibly a CpG containing oligonucleotide. In one aspect, said CpG containing oligonucleotide comprises a Purine, Purine, C,G, pyrimidine, pyrimidine sequence. In another aspect, said immunostimulatory oligonucleotide is selected from the group consisting of: SEQ ID NO:1; SEQ ID NO:2; SEQ ID NO:3; SEQ ID NO:4; and SEQ ID NO:5.

Whilst not wishing to be bound by theory it is thought that providing the antigen and the TLR9 agonist together provides a component that is more stable than simply the addition of the TLR9 to a liquid formulation of MPL and QS21.

The present invention provides the advantage that where the antigen and TLR9 agonist are reconstituted with WFI one is able to provide only one vial with lyophilized formulation in it. Furthermore, where the antigen and the TRL9 agonist are to be reconstituted with a liquid formulation such as a liquid adjuvant formulation then it is advantageous to be able to provide only two vials of components (rather than three). This in turn has cost benefits,whilst providing a product suitable for use a vaccine once reconstituted.

Furthermore, the present inventors have found that the co-lyophilisation of CpG with antigens which would not have an overall positive charge in the reconstitution buffer may increase the solubility of those antigens on reconstitution with either water for injection or liquid adjuvant. Therefore the present invention also provides a method to increase the solubility of a lyophilised antigen on reconstitution where the antigen would not have a net positive charge in the reconstitution buffer comprising the step of co-lyophilising a TLR9 agonist, preferably an immunostimulatory oligonucleotide and more preferably a CpG oligonucleotide with the antigen. The present invention also provides for the use of a TLR9 agonist, preferably an immunostimulatory oligonucleotide and more preferably a CpG oligonucleotide to increase the solubility of a lyophilised non-positively charged antigen on reconstitution. By "non-positively charged" is meant that the overall charge of the protein is not positive. The protein may contain both positive and negative charges, but the overall charge of the protein is either neutral or negative.

The present invention also provides a method of making an immunogenic composition comprising the steps of reconstituting a lyophilised composition as described herein with a suitable carrier. In one embodiment, said carrier is a liposomal solution or an oil in water emulsion. Said carrier may optionally contain one or more immunostimulants, which may be selected from the group consisting of TLR4 agonists, TLR4 antagonists, saponins, TLR7 agonists, TLR8 agonists, TLR9 agonists. In one embodiment, said carrier contains two or more immunostimulants and in one aspect these may be 3-deacylated MPL and QS21.

The present invention also provides a method of making a lyophilised composition of the invention comprising combining one or more desired antigens, a TLR9 ligand and suitable excipients and freeze drying the resulting mixture.

**Detailed Description of the Invention**

The present inventors have found that TLR9 ligands such a CpG oligonucleotides may be lyophilised with an antigen of interest without affecting the antigenicity or stability of that antigen. By TLR9 ligand is meant a compound that can interact with the TLR9 receptor.

Members of the Toll-Like Receptor (TLR) family, first discovered in Drosophila, have been shown to be pattern recognition receptors, each member recognizing and responding to different microbial components to limit/eradicate invading microbes. Binding of pathogen-associated molecular patterns (PAMP) to TLRs induces the production of reactive oxygen and nitrogen intermediates, initiation of the pro-inflammatory cytokine network, and upregulation of costimulatory molecules linking the rapid innate response to the adaptive immunity. Many TLR ligands are known to be useful as adjuvants. TLR9 has been shown to respond to oligonucleotide agonists. Therefore the TLR9 ligands of the invention are immunostimulatory oligonucleotides. In one embodiment of the invention, such TLR9 ligands contain a CpG motif. Alternative immunostimulatory oligonucleotides may comprise modifications to the nucleotides. For example, WO0226757 and WO03057822 disclose modifications to the C and G portion of a CpG containing immunostimulatory oligonucleotides.

In one embodiment, the TLR9 ligands are CpG oligonucleotides. In one aspect of this embodiment, a CpG oligonucleotide contains two or more dinucleotide CpG motifs separated by at least three, possibly at least six or more nucleotides. The oligonucleotides of the present invention are typically deoxynucleotides. In one embodiment the internucleotide bond in the oligonucleotide is phosphorodithioate, or possibly a phosphorothioate bond, although phosphodiester and other internucleotide bonds could also be used, including oligonucleotides with mixed internucleotide linkages. Methods for producing phosphorothioate oligonucleotides or phosphorodithioate are described in US5,666,153, US5,278,302 and WO95/26204. Oligonucleotide comprising different internucleotide linkages are contemplated, e.g. mixed phosphorothioate phophodiesters. Other internucleotide bonds which stabilise the oligonucleotide may be used.

Examples of CpG oligonucleotides have the following sequences. In one embodiment, these sequences contain phosphorothioate modified internucleotide linkages.

OLIGO 1(SEQ ID NO:1): TCC ATG ACG TTC CTG ACG TT (CpG 1826)
OLIGO 2 (SEQ ID NO:2): TCT CCC AGC GTG CGC CAT (CpG 1758)
OLIGO 3(SEQ ID NO:3): ACC GAT GAC GTC GCC GGT GAC GGC ACC ACG
OLIGO 4 (SEQ ID NO:4): TCG TCG TTT TGT CGT TTT GTC GTT (CpG 2006)
OLIGO 5 (SEQ ID NO:5): TCC ATG ACG TTC CTG ATG CT (CpG 1668)

Alternative CpG oligonucleotides may comprise the sequences above in that they have inconsequential deletions or additions thereto.

The CpG oligonucleotides utilised in the present invention may be synthesized by any method known in the art (eg EP 468520). Conveniently, such oligonucleotides may be synthesized utilising an automated synthesizer.

In the context of the present specification, the term "antigen" is intended to refer to an immunogenic component suitable for raising a specific immune response and suitable for

inclusion into to a vaccine or immunogenic composition, for example an antigen for inclusion in a HIV-1 vaccine, a cancer vaccine, a malaria vaccine, a TB vaccine or the like. Details of specific antigens are given below.

In one embodiment the antigen has an isoelectric point of 9.6 or less. In one embodiment the antigen has isoelectric point of 9 or less. In one embodiment the antigen has an isoelectric point of 8.5 or less. In one embodiment the antigen has an isoelectric point of 8.0 or less. In one embodiment the antigen has an isoelectric point of 7.5. In one embodiment the antigen has an isoelectric point in the range 7 to 8.

The net charge of a protein when reconstituted in buffer depends on the number of positive versus the number of negative charges in the protein, this charge will of course vary depending on the pH of the reconstitution buffer Isoelectric point is the pH at which the net charge of a protein is neutral. If the pH of the reconstitution buffer is below the isoelctric point of the antigen, the protein tends to carry a net positive charge. If the pH of the reconstitution buffer is above the isoelectric point of the antigen, the protein tends to carry a net negative charge. The present invention is particularly useful when lyophilising and reconstituting antigens which have an isoelectric point such that, in the intended reconstitution buffer, the protein would carry a net negative charge. In such circumstances (see example 3), the presence of CpG in the lyophilised composition can enhance solubility of the antigen in the reconstitution buffer.

In one embodiment the lyophilized antigen and TLR9 agonist is provided as one dose, for example in one vial.

In one embodiment the lyphilized antigen is present in an amount to provide an antigen concentration in the range of 10 to 250 µg, when reconstituted.

In one embodiment the TRL9 agonist is present in an amount to provide a concentration in the range of 10 to 1000 µg such as 500µg, when reconstituted.

In one embodiment of the invention, the antigen which is combined in a lyophilised composition with a TLR9 ligand may be an anti- tumour antigen. Therefore immunogenic compositions made using the lyophilised antigenic composition of the invention are useful for the immunotherapeutic treatment of cancers. For example, lyophilised composition may be prepared with cancer antigens, tumour antigens or tumour rejection antigens as described herein, such as those
proteins expressed in prostate cancer, breast cancer, colorectal cancers, lung cancer, kidney cancer, ovarian cancer, liver cancer and head and neck cancer, among others.

Cancer testis antigens that may be used in the present invention include the MAGE A family of antigens MAGE-A1, A2, A3, A4, A5, A6, A7, A8, A9, A10, A11 and A12; also known as MAGE-1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12), the MAGE B antigens MAGE B1, B2,

B3 and B4, the MAGE C antigens MAGE-C1 and MAGE-C2 , the LAGE 1 antigen, the LAGE 2 antigen (also known as NY-ESO-1) and the GAGE antigen.

Prostate specific antigens may also be used in the present invention. Examples of prostate specific antigens that may be fused include six-transmembrane epithelial antigen of the prostate (STEAP), Prostate Specific Antigen (PSA), prostatic acid phosphatase (PAP), prostate stem cell antigen (PSCA), prostate-specific membrane antigen (PSMA) or the antigen known as prostase (also known as P703P).

In one embodiment, the prostate antigen is P501S or a fragment thereof. P501S, also named prostein, is a 553 amino acid protein. Immunogenic fragments and portions of P501S comprising at least 20, 50, or 100 contiguous amino acids, or fragments comprising between 20-50 or 50-100 contiguous amino acids, may be used as the tumour associated antigen or derivative of the present invention. In one embodiment the tumour associated antigen or derivative is the PS108 antigen (disclosed in WO98/50567) or prostate cancer-associated protein (see WO99/67384). In some embodiments, fragments are amino acids 51-553, 34-553 or 55-553 of the full-length P501S protein. These can be expressed in yeast systems, for example DNA sequences encoding such polypeptides can be expressed in yeast systems.

In one embodiment, the antigen may comprise or consist of WT-1 expressed by the Wilm's tumor gene, or its N-terminal fragment WT-1F comprising about or approximately amino acids 1-249. WT1 is a protein originally found to be overexpressed in paediatric kidney cancer, Wilm's Tumor. An antigen that may be used comprises nearly the full length protein as antigen. In one embodiment, the antigen may comprise or consist of the WT1-A10 protein, which is a 292 AA recombinant fusion protein consisting of a 12mer truncated tat sequence and amino acids number 2 – 281 of the WT1 sequence.

In one embodiment of the invention the tumour associated antigen or derivative is a breast cancer antigen, for example Her-2/neu, mammaglobin or a B305D antigen.

The Her-2/neu antigen for use in the present invention may comprises the entire extracellular domain (ECD; for example the sequence comprising approximately amino acid 1–645 of the amino acid sequence of Her-2/neu) or fragments thereof. Alternatively or additionally the construct may comprise at least an immunogenic portion of or the entire intracellular domain: for example approximately the C terminal 580 amino acids of the Her-2/neu sequence.

One construct that may be used as the tumour associated antigen derivative of the present invention is a fusion protein of the ECD and the phosphorylation domain (PD) of Her-2/neu (ECD-PD). A further construct that may be used is a fusion protein of the ECD and a fragment of the phosphorylation domain of Her-2/neu (ECD-ΔPD). The Her-2/neu fusion proteins and constructs as described may be derived from human, rat, mouse or simian/monkey Her-2/neu. Exemplary sequences and constructs of Her-2/neu are described in WO00/44899.

PRAME (also known as DAGE) is another antigen that may be used as the tumour associated antigen of the present invention. Fusion proteins as described herein that comprise the PRAME antigen may also be used. In particular, fusions of the PRAME antigen as described herein and a protein D fusion partner protein or derivative as described herein are contemplated for use in the present invention.

PRAME antigen has been shown by some groups to be expressed in melanoma and a wide variety of tumours including lung, kidney and head and neck cancer. Interestingly it also seems to be expressed in 40-60% leukemia such as acute lymphoid leukemia and acute myeloid leukemia, see for example Exp Hematol. 2000 Dec;28(12):1413-22. In patients it has been observed that over expression of PRAME seems to be associated with higher survival and lower rates of relapse in comparison to those who do not over express the protein.

The antigen and its preparation are described in US patent No. 5, 830, 753. PRAME is found in the Annotated Human Gene Database H-Inv DB under the accession numbers: U65011.1, BC022008.1, AK129783.1, BC014974.2, CR608334.1, AF025440.1, CR591755.1, BC039731.1, CR623010.1, CR611321.1, CR618501.1, CR604772.1, CR456549.1, and CR620272.1.

In one aspect the antigen of the present invention may comprise or consist of a PRAME antigen or immunogenic fragment thereof. Generally the PRAME protein has 509 amino acids and in one embodiment all 509 amino acids of PRAME may be included in the antigen.

Colorectal antigens may also be used as the tumour associated antigens of the present invention. Examples of colorectal antigens that could be used include: C1585P (MMP 11)

and C1491 (E1A Enhancer Binding Protein), CASB618 (as described in WO00/53748); CASB7439 (as described in WO01/62778); and C1584 (Cripto).

Other tumour associated antigens useful in the context of the present invention include: Plu –1 J Biol. Chem 274 (22) 15633 –15645, 1999, HASH –1, HASH-2, Cripto (Salomon et al Bioessays 199, 21 61 –70,US patent 5654140) Criptin US patent 5 981 215. Additionally, antigens particularly relevant for vaccines in the therapy of cancer also comprise tyrosinase and survivin.

Mucin dervied peptides such as Muc1 see for example US 5744,144 US 5827, 666 WO 8805054, US 4,963,484. Specifically contemplated are Muc 1 derived peptides that comprise at least one repeat unit of the the Muc 1 peptide, preferably at least two such repeats and which is recognised by the SM3 antibody (US 6 054 438). Other mucin derived peptides include peptide from Muc 5.

Other tumour-specific antigens are suitable for use in the lyophillised composition of the present invention and include, but are not restricted to tumour-specific gangliosides such as GM 2, and GM3 or conjugates thereof to carrier proteins; or said antigen may be a self peptide hormone such as whole length Gonadotrophin hormone releasing hormone (GnRH, WO 95/20600), a short 10 amino acid long peptide, useful in the treatment of many cancers, or in immunocastration.

The invention also extends to use of the above antigens, immunogenic derivatives and immunogenic fragments and fusion proteins comprising same in aspects of the present invention.

**Derivatives, fragments and fusion proteins**

Tumour associated antigens of the present invention may be employed in the form of derivatives or fragments thereof rather than the naturally-occurring antigen.

As used herein the term “derivative” refers to an antigen that is modified relative to its naturally occurring form. The derivative may include a mutation, for example a point mutation. In one example, the derivative may change the properties of the protein, for example by improving expression in prokaryotic systems or by removing undesirable activity,, e.g., enzymatic activity. Derivatives of the present invention are sufficiently similar to native antigens to retain antigenic properties and remain capable of allowing an immune response to be raised against the native antigen. Whether or not a given derivative raises such an immune response may be measured by a suitably immunological assay such as an ELISA or flow cytometry.

In one embodiment of the present invention the derivative of the tumour associated antigen of the present invention is a fusion protein comprising a tumour associated antigen linked to a heterologous fusion partner protein. By "heterologous" with respect to a tumour associated antigen is intended a protein or polypeptide sequence that would not be linked to the tumour associated antigen in nature, i.e., is linked to the tumour associated antigen by deliberate human intervention.

The antigen and heterologous fusion partner protein may be chemically conjugated or may be expressed as recombinant fusion proteins. In one embodiment, a fusion protein of the present invention may allow increased levels of the fusion protein to be produced in an expression system compared to non-fused protein. Thus the fusion partner protein may assist in providing T helper epitopes, for example T helper epitopes recognised by humans (ie. the fusion partner protein is acting as an immunological fusion partner). The fusion partner may assist in expressing the protein at higher yields than the native recombinant protein (i.e., the fusion partner protein acting as an expression enhancer). In one embodiment, the fusion partner protein may act as both an immunological fusion partner and expression enhancing partner.

Fusion partner proteins may, for example, be derived from protein D. Protein D is a lipoprotein (a 42 kDa immunoglobulin D binding protein exposed on the surface of the Gram-negative bacterium Haemophilus influenzae). The protein is synthesized as a precursor with an 18 amino acid residue signal sequence, containing a consensus sequence for bacterial lipoprotein (see WO 91/18926). Native precursor Protein D protein is processed during secretion and the signal sequence is cleaved. The Cys of the processed Protein D (at position 19 in the precursor molecule) becomes the N terminal residue of the processed protein and is concomitantly modified by covalent attachment of both ester-linked and amide-linked fatty acids. The fatty acids linked to the amino-terminal Cysteine residue then function as membrane anchor.

In one embodiment, the tumour associated antigen derivative for use in the present invention may comprise Protein D or a derivative thereof as a fusion partner protein.

The protein D or a derivative thereof as described herein may comprise, for example: the first or N-terminal third of processed protein D or approximately or about the first or N-terminal third of processed protein D. In one embodiment, the protein D or a derivative

thereof may comprise the first or N-terminal 100 to 115 amino acids of processed protein D; or the first or N-terminal 109 amino acids of processed protein D. In one embodiment, the native processed Protein D amino acids 2-Lys and 2-Leu may be substituted with amino acids 2-Asp and 3-Pro.

In one embodiment, the protein D or derivative thereof may further include the 18 or 19 amino acid signal sequence of precursor protein D. In one embodiment, the fusion partner protein derived from protein D comprises or consists of amino acids 20 to 127 of precursor protein D. In one embodiment of the present invention, the two amino acids 21-Lys and 22-Leu of the precursor protein D fusion partner protein may be substituted with amino acids 21-Asp and 22-Pro.

The protein D fusion partner protein as described herein may additionally or alternatively contain deletions, substitutions or insertions within the amino acid sequence when compared to the wild-type precursor or processed protein D sequence. In one embodiment, 1, 2, 3, 4, 5, 6, 7, 8, 9 or more amino acids may be inserted, substituted or deleted. The amino acids may be substituted with conservative substitutions as defined herein, or other amino acids may be used.

In one embodiment, the fusion partner protein may comprise or consist of a protein D sequence as shown in SEQ ID NO: 1. In one embodiment, the fusion partner protein may comprise or consist of the amino acids underlined in Figure 1, ie., amino acid residues 20 through 127 of SEQ ID NO: 12. In one embodiment, the antigen for use in the present invention may be protein-D-MAGE-3, in which the MAGE-3 antigen consists of amino acids 3 to 314 of MAGE-3 and in which the protein D fusion partner protein consists of the amino acid sequence shown in Figure 1.

In another embodiment of the present invention, fusion partner proteins may be selected from NS1 or LytA or derivatives thereof as described below.

NS1 is a non-structural protein from the influenzae virus. In one embodiment, the tumour associated antigen derivative of the present invention may comprise NS1 or a derivative thereof as a fusion partner protein. The NS1 or derivative thereof may comprise the N terminal 1 to 81 amino acids thereof.

LytA is derived from Streptococcus pneumoniae. The C-terminal domain of the LytA protein is responsible for the affinity to the choline or to some choline analogues such as DEAE. In one embodiment, the tumour associated antigen derivative of the present invention may comprise LytA or a derivative thereof as a fusion partner protein. The LytA or derivative thereof may comprise the repeat portion of the LytA molecule found in the C terminal end starting at residue 178. In one embodiment, the LytA or derivative thereof comprises residues 188 – 305 of C-LytA.

Immunogenic polypeptides for use in the present invention will typically be recombinant proteins produced, e.g., by expression in a heterologous host such as a bacterial host, in yeast or in cultured mammalian cells.

The term "tumor associated antigen derivative" means a polypeptide which partially or wholly contains sequences which occur naturally in a tumor associated antigen or which bears a high degree of sequence identity thereto (e.g., more than 95% identity over a stretch of at least 10, e.g., at least 20 amino acids). Derivatives also include sequences having conservative substituitions. Conservative substitutions are well known and are generally set up as the default scoring matrices in sequence alignment computer programs.

In general terms, substitution within the following groups are conservative substitutions, but substitutions between the following groups are considered non-conserved. The groups are:

i) Aspartate/asparagine/glutamate/glutamine
ii) Serine/threonine
iii) Lysine/arginine
iv) Phenylalanine/tyrosine/tryptophane
v) Leucine/isoleucine/valine/methionine
vi) Glycine/alanine

Derivatives of the present invention may also include chemically treated sequences such as treatment with an aldehyde (such as formaldehyde or glutaraldehyde), carboxymethylation, carboxyamidation, acetylation and other routine chemical treatments. Constructs of the present invention having derivatised free thiol residues may also be

used in the present invention. In particular carboxyamidated or carboxymethylated thiol derivatives may be used.

In one embodiment of the present invention the tumor associated antigen derivative may be a MAGE antigen as described herein having derivatised free thiol residues. The derivatised free thiol residues may be a carboxyamide or carboxymethylated derivatives.

The tumour associated antigen derivative of the present invention may alternatively comprise a construct comprising more than one tumour associated antigen. In one embodiment of the present invention, the tumour associated antigen derivative may comprise two or more tumour associated antigens.

The term "fragment" as used herein refers to fragments of a tumour associated antigen or derivative of the antigen which contain at least one epitope, for example a CTL epitope, typically a peptide of at least 8 amino acids. Fragments of at least 8, for example 8-10 amino acids or up to 20, 50, 60, 70, 100, 150 or 200 amino acids in length are considered to fall within the scope of the invention as long as the fragment demonstrates antigenicity, that is to say that the major epitopes (e.g., CTL epitopes) are retained by the fragment and the fragment is capable of inducing an immune response that cross-reacts with the naturally occurring tumour associated antigen. Exemplary fragments may be 8-10, 10-20, 20-50, 50-60, 60-70, 70-100, 100-150, 150-200 amino acid residues in length (inclusive of any value within these ranges).

In one embodiment of the invention, the lyophilised composition comprising Her 2 neu antigen and CpG oligonucleotide is reconstituted with a liposome or oil in water emulsion carrier containing 3D-MPL and QS21. Such reconstituted formulations produce both a humoral and cellular mediated response.

The lyophilised compositions of the invention may contain antigens associated with tumour-support mechanisms (e.g. angiogenesis, tumour invasion) for example tie 2, VEGF.

In another aspect of the invention, the antigen within the lyophilised composition of the invention is an antigen selected from HIV derived antigens, particularly HIV-1 derived antigens. The following passages describe the antigens which may be derived from HIV-1.

HIV Tat and Nef proteins are early proteins, that is, they are expressed early in infection and in the absence of structural protein.

The Nef gene encodes an early accessory HIV protein which has been shown to possess several activities. For example, the Nef protein is known to cause the removal of CD4, the HIV receptor, from the cell surface, although the biological importance of this function is debated. Additionally Nef interacts with the signal pathway of T cells and induces an active state, which in turn may promote more efficient gene expression. Some HIV isolates have mutations or deletions in this region, which cause them not to encode functional protein and are severely compromised in their replication and pathogenesis *in vivo*.

The Gag gene is translated from the full-length RNA to yield a precursor polyprotein which is subsequently cleaved into 3 – 5 capsid proteins; the matrix protein p17, capsid protein p24 and nucleic acid binding protein (Fundamental Virology, Fields BN, Knipe DM and Howley M 1996 2. Fields Virology vol 2 1996).

The Gag gene gives rise to the 55-kilodalton (Kd) Gag precursor protein, also called p55, which is expressed from the unspliced viral mRNA. During translation, the N terminus of p55 is myristoylated, triggering its association with the cytoplasmic aspect of cell membranes. The membrane-associated Gag polyprotein recruits two copies of the viral genomic RNA along with other viral and cellular proteins that triggers the budding of the viral particle from the surface of an infected cell. After budding, p55 is cleaved by the virally encoded protease (a product of the Pol gene) during the process of viral maturation into four smaller proteins designated MA (matrix [p17]), CA (capsid [p24]), NC (nucleocapsid [p9]), and p6.

In addition to the 3 major Gag proteins (p17, p24 and p9), all Gag precursors contain several other regions, which are cleaved out and remain in the virion as peptides of various sizes. These proteins have different roles e.g. the p2 protein has a proposed role in regulating activity of the protease and contributes to the correct timing of proteolytic processing.

The MA polypeptide is derived from the N-terminal, myristoylated end of p55. Most MA molecules remain attached to the inner surface of the virion lipid bilayer, stabilizing the particle. A subset of MA is recruited inside the deeper layers of the virion where it becomes part of the complex which escorts the viral DNA to the nucleus. These MA molecules facilitate the nuclear transport of the viral genome because a karyophilic signal on MA is recognized by the cellular nuclear import machinery. This phenomenon allows HIV to infect non-dividing cells, an unusual property for a retrovirus.

The p24 (CA) protein forms the conical core of viral particles. Cyclophilin A has been demonstrated to interact with the p24 region of p55 leading to its incorporation into HIV particles. The interaction between Gag and cyclophilin A is essential because the disruption of this interaction by cyclosporine inhibits viral replication.

The NC region of Gag is responsible for specifically recognizing the so-called packaging signal of HIV. The packaging signal consists of four stem loop structures located near the

5' end of the viral RNA, and is sufficient to mediate the incorporation of a heterologous RNA into HIV-1 virions. NC binds to the packaging signal through interactions mediated by two zinc-finger motifs. NC also facilitates reverse transcription.

The p6 polypeptide region mediates interactions between p55 Gag and the accessory protein Vpr, leading to the incorporation of Vpr into assembling virions. The p6 region also contains a so-called late domain which is required for the efficient release of budding virions from an infected cell.

The Pol gene encodes three proteins having the activities needed by the virus in early infection, reverse transcriptase RT, protease, and the integrase protein needed for integration of viral DNA into cellular DNA. The primary product of Pol is cleaved by the virion protease to yield the amino terminal RT peptide which contains activities necessary for DNA synthesis (RNA and DNA directed DNA polymerase, ribonuclease H) and carboxy terminal integrase protein. HIV RT is a heterodimer of full-length RT (p66) and a cleavage product (p51) lacking the carboxy terminal RNase H domain.

RT is one of the most highly conserved proteins encoded by the retroviral genome. Two major activities of RT are the DNA Pol and ribonuclease H. The DNA Pol activity of RT uses RNA and DNA as templates interchangeably and like all DNA polymerases known is unable to initiate DNA synthesis de novo, but requires a pre existing molecule to serve as a primer (RNA).

The RNase H activity inherent in all RT proteins plays the essential role early in replication of removing the RNA genome as DNA synthesis proceeds. It selectively degrades the RNA from all RNA - DNA hybrid molecules. Structurally the polymerase and ribo H occupy separate, non-overlapping domains within the Pol covering the amino two thirds of the Pol.

The p66 catalytic subunit is folded into 5 distinct subdomains. The amino terminal 23 of these have the portion with RT activity. Carboxy terminal to these is the RNase H domain.

After infection of the host cell, the retroviral RNA genome is copied into linear double stranded DNA by the reverse transcriptase that is present in the infecting particle. The integrase (reviewed in Skalka AM '99 Adv in Virus Res 52 271-273) recognises the ends of the viral DNA, trims them and accompanies the viral DNA to a host chromosomal site to catalyse integration. Many sites in the host DNA can be targets for integration. Although the integrase is sufficient to catalyse integration in vitro, it is not the only protein associated with the viral DNA in vivo - the large protein - viral DNA complex isolated from the infected cells has been denoted the pre integration complex. This facilitates the acquisition of the host cell genes by progeny viral genomes.

The integrase is made up of 3 distinct domains, the N terminal domain, the catalytic core and the C terminal domain. The catalytic core domain contains all of the requirements for the chemistry of polynucleotidyl transfer.

HIV-1 derived antigens for use in the invention may thus for example be selected from Gag (for example full length Gag), p17 (a portion of Gag), p24 (another portion of Gag), p41, p40, Pol (for example full lemgth Pol), RT (a portion of Pol), p51 (a portion of RT), integrase (a portion of Pol), protease (a portion of Pol), Env, gp120, gp140 or gp160, gp41, Nef, Vif, Vpr, Vpu, Rev, Tat and immunogenic derivatives thereof and immunogenic fragments thereof, particularly Env, Gag, Nef and Pol and immunogenic derivatives thereof and immunogenic fragments thereof including p17, p24, RT and integrase. HIV vaccines may comprise polypeptides and/or polynucleotides encoding polypeptides corresponding to multiple different HIV antigens for example 2 or 3 or 4 or more HIV antigens which may be selected from the above list. Several different antigens may, for example, be comprised in a single fusion protein. More than one first immunogenic polypeptide and/or more than one second immunogenic polypeptide each of which is an HIV antigen or a fusion of more than one antigen may be employed.

For example an antigen may comprise Gag or an immunogenic derivative or immunogenic fragment thereof, fused to RT or an immunogenic derivative or immunogenic fragment thereof, fused to Nef or an immunogenic derivative or immunogenic fragment thereof wherein the Gag portion of the fusion protein is present at the 5' terminus end of the polypeptide.

A Gag sequence of use according to the invention may exclude the Gag p6 polypeptide encoding sequence. A particular example of a Gag sequence for use in the invention comprises p17 and/or p24 encoding sequences.

A RT sequence may contain a mutation to substantially inactivate any reverse transcriptase activity (see WO03/025003).

The RT gene is a component of the bigger *pol* gene in the HIV genome. It will be understood that the RT sequence employed according to the invention may be present in the context of Pol, or a fragment of Pol corresponding at least to RT. Such fragments of Pol retain major CTL epitopes of Pol. In one specific example, RT is included as just the p51 or just the p66 fragment of RT.

The RT component of the fusion protein or composition according to the invention optionally comprises a mutation to remove a site which serves as an internal initiation site in prokaryotic expression systems.

Optionally the Nef sequence for use in the invention is truncated to remove the sequence encoding the N terminal region i.e. removal of from 30 to 85 amino acids, for example from 60 to 85 amino acids, particularly the N terminal 65 amino acids (the latter truncation is referred to herein as trNef). Alternatively or additionally the Nef may be modified to

remove the myristylation site. For example the Gly 2 myristylation site may be removed by deletion or substitution. Alternatively or additionally the Nef may be modified to alter the dileucine motif of Leu 174 and Leu 175 by deletion or substitution of one or both leucines. The importance of the dileucine motif in CD4 downregulation is described e.g. in Bresnahan P.A. et al (1998) Current Biology, 8(22): 1235-8.

The Env antigen may be present in its full length as gp160 or truncated as gp140 or shorter (optionally with a suitable mutation to destroy the cleavage site motif between gp120 and gp41). The Env antigen may also be present in its naturally occurring processed form as gp120 and gp41. These two derivatives of gp160 may be used individually or together as a combination. The aforementioned Env antigens may further exhibit deletions (in particular of variable loops) and truncations. Fragments of Env may be used as well.

An exemplary gp120 sequence is shown in SEQ ID No 6. An exemplary gp140 sequence is shown in SEQ ID No 7.

Immunogenic polypeptides for use in a lyophilised composition according to the invention may comprise Gag, Pol, Env and Nef wherein at least 75%, or at least 90% or at least 95%, for example, 96% of the CTL epitopes of these native antigens are present.

In lyophilised compositions comprising immunogenic polypeptides which comprise p17/p24 Gag, p66 RT, and truncated Nef as defined above, 96% of the CTL epitopes of the native Gag, Pol and Nef antigens are suitably present.

One embodiment of the invention provides a lyophilised composition comprising a TLR9 ligand and an immunogenic polypeptide containing p17, p24 Gag, p66 RT , truncated Nef (devoid of nucleotides encoding terminal amino-acids 1-85 – "trNef") in the order Gag, RT, Nef.

Specific polynucleotide constructs and corresponding polypeptide antigens for use in lyophilised compositions according to the invention include:

1. p17, p24 (codon optimised) Gag - p66 RT (codon optimised) - truncated Nef;
2. truncated Nef - p66 RT (codon optimised) - p17, p24 (codon optimised) Gag;
3. truncated Nef - p17, p24 (codon optimised) Gag - p66 RT (codon optimised);
4. p66 RT (codon optimised) - p17, p24 (codon optimised) Gag - truncated Nef;
5. p66 RT (codon optimised) – truncated Nef - p17, p24 (codon optimised) Gag;
6. p17, p24 (codon optimised) Gag - truncated Nef - p66 RT (codon optimised).

An exemplary fusion is a fusion of Gag, RT and Nef particularly in the order Gag-RT-Nef (see eg SEQ ID No 8 or SEQ ID NO: 9) Another exemplary fusion is a fusion of p17, p24, RT and Nef particularly in the order p24-RT-Nef-p17. This fusion is called F4 and is described in WO2006/013106. F4 is a preferred example of an HIV antigen which may be found in a lyophilised composition of the invention. The nucleotide sequence of F4 is given in SEQ ID NO:10 where p24 sequence is in bold, the Nef sequence is underlined,

and the boxes are nucleotides introduced by genetic construction. The amino acid sequence of F4 is given in SEQ ID NO:11, where:
P24 sequence: amino-acids 1-232 (in bold)
RT sequence: amino-acids 235-795
Nef sequence: amino-acids 798-1002
P17 sequence: amino-acids 1005-1136
Boxes:amino-acids introduced by genetic construction
K (Lysine): instead of Tryptophan (W). Mutation introduced to remover enzyme activity

In another embodiment a lyophilised composition contains Gag, RT, integrase and Nef, especially in the order Gag-RT-integrase-Nef (see eg SEQ ID No 11).

In other embodiments the HIV antigen may be a fusion polypeptide which comprises Nef or an immunogenic derivative thereof or an immunogenic fragment thereof, and p17 Gag and/or p24 Gag or immunogenic derivatives thereof or immunogenic fragments thereof, wherein when both p17 and p24 Gag are present there is at least one HIV antigen or immunogenic fragment between them.

For example, Nef is suitably full length Nef.

For example p17 Gag and p24 Gag are suitably full length p17 and p24 respectively.

In one embodiment a lyophilised composition contains an immunogenic polypeptide comprising both p17 and p24 Gag or immunogenic fragments thereof. In such a construct the p24 Gag component and p17 Gag component are separated by at least one further HIV antigen or immunogenic fragment, such as Nef and/or RT or immunogenic derivatives thereof or immunogenic fragments thereof. See WO2006/013106 for further details.

In fusion proteins which comprise p24 and RT, it may be preferable that the p24 precedes the RT in the construct because when the antigens are expressed alone in *E. coli* better expression of p24 than of RT is observed.

Some constructs for use in lyophilised compositions according to the invention include the following:

1. p24 – RT – Nef – p17
2. p24 – RT* – Nef – p17
3. p24 – p51RT – Nef – p17
4. p24 – p51RT* - Nef – p17
5. p17 – p51RT – Nef
6. p17 – p51RT* - Nef
7. Nef – p17
8. Nef – p17 with linker
9. p17 – Nef
10. p17 – Nef with linker

* represents RT methionine$_{592}$ mutation to lysine

In another aspect the present invention provides a lyophilised composition containing a fusion protein of HIV antigens comprising at least four HIV antigens or immunogenic fragments, wherein the four antigens or fragments are or are derived from Nef, Pol and Gag. Preferably Gag is present as two separate components which are separated by at least one other antigen in the fusion. Preferably the Nef is full length Nef. Preferably the Pol is p66 or p51RT. Preferably the Gag is p17 and p24 Gag. Other preferred features and properties of the antigen components of the fusion in this aspect of the invention are as described herein.

Preferred embodiments of this aspect of the invention are the four component fusions as already listed above:

1. p24 – RT – Nef – p17
2. p24 – RT* – Nef – p17
3. p24 – p51RT – Nef – p17
4. p24 – p51RT* - Nef – p17

The immunogenic polypeptides used within the lyophilised composition of the present invention may have linker sequences present in between the sequences corresponding to particular antigens such as Gag, RT and Nef. Such linker sequences may be, for example, up to 20 amino acids in length. In a particular example they may be from 1 to 10 amino acids, or from 1 to 6 amino acids, for example 4-6 amino acids.

Further description of such suitable HIV antigens can be found in WO03/025003.

HIV antigens for use in the present invention may be derived from any HIV clade, for example clade A, clade B or clade C. For example the HIV antigens may be derived from clade A or B, especially B.

In one specific embodiment of the invention, a lyophilised composition contains more than one immunogenic polypeptide. In one aspect of this embodiment a first immunogenic polypeptide is a polypeptide comprising Gag and/or Pol and/or Nef or a fragment or derivative of any of them (eg p24-RT-Nef-p17) . In one specific aspect of this embodiment of the invention a second immunogenic polypeptide is a polypeptide comprising Gap and/or Pol and/or Nef or a fragment or derivative of any of them (eg Gag-RT-Nef or Gag-RT-integrase-Nef).

Thus in one specific embodiment, a polypeptide comprising Gap and/or Pol and/or Nef or a fragment or derivative of any of them (eg p24-RT-Nef-p17) is a first immunogenic polypeptide and a polypeptide comprising Gap and/or Pol and/or Nef or a fragment or derivative of any of them (eg Gag-RT-Nef or Gag-RT-integrase-Nef) is a second immunogenic polypeptide.

In another specific embodiment of the invention, a first immunogenic polypeptide is Env or a fragment or derivative thereof eg gp120, gp140 or gp160 (especially gp120). In one specific embodiment of the invention a second immunogenic polypeptide is a polypeptide

comprising Gag and/or Pol and/or Nef or a fragment or derivative of any of them (eg p24-RT-Nef-p17).

Thus in one specific embodiment, Env or a fragment or derivative thereof eg gp120, gp140 or gp160 (especially gp120) is a first immunogenic polypeptide and a polypeptide comprising Gag and/or Pol and/or Nef or a fragment or derivative of any of them (eg p24-RT-Nef-p17) is a second immunogenic polypeptide.

In another specific embodiment of the invention, a first immunogenic polypeptide is a polypeptide comprising Gag and/or Pol and/or Nef or a fragment or derivative of any of them (eg p24-RT-Nef-p17). In one specific embodiment of the invention a second immunogenic polypeptide is Env or a fragment or derivative thereof eg gp120, gp140 or gp160 (especially gp120).

Thus in one specific embodiment, a polypeptide comprising Gag and/or Pol and/or Nef or a fragment or derivative of any of them (eg p24-RT-Nef-p17) is a first immunogenic polypeptide and Env or a fragment or derivative thereof eg gp120, gp140 or gp160 (especially gp120) is a second immunogenic polypeptide.

The lyophilised composition may contain one antigen, or may contain more than one antigen.

In one aspect of the invention, the TLR9 ligand is used to improve the solubility of non-positively charged antigens. The present inventors have found that, particularly with antigens which are negatively charged, the co-lyophilisation of Cpg can improve their solubility on reconstitution. Where the TLR9 ligand is an immunostimulatory oligonucleotide, the antigen will be a molecule with a net negative charge. Where this ligand is co-lyophilised with an antigen with a net positive charge, there is a possibility that the TLR9 ligand will interact with the antigen upon reconstitution of the lyophilised composition, possibly causing precipitation of the antigen. This is not desirable, but can be avoided by one of skill in the art by including with the composition for lyophilisation excipients which are known to increase solubility in such situations such as, for example, L-arginine.

The TLR9 ligand and one or more antigens are combined with suitable excipients to form the final bulk formulation which will be lyophilised. Optimally, the excipients will contain a cryoprotectant to protect the protein from denaturation during the early stages of lyophilisation, and a lyoprotectant to prevent protein inactivation during drying. Two different molecules may be used, or one molecule may be used that has both properties, such as a disaccharide. Optionally, a crystalline bulking agent such as mannitol or glycine may also be added. A non-ionic surfactant such as polysorbate or Tween® may also be added to help prevent aggregation of the protein. Excipients could also include buffer salts to modify the pH of the final bulk.

Suitable excipients include the following: sugars such as sucrose, trehalose, raffinose and maltodextrins such as maltotriose, maltotetraose, maltopentaose or maltohexaose; polyols such as mannitol or sorbitol; polymers such as dextran, polyethylene glycol (PEG), or polyvinylpyrrolidone (PVP); amino acids such as glycine, alanine or arginine.

Excipients may also be combined such that two or more, for example three or four excipients may be used together. Possible combinations include sugar and dextran, for example sucrose and dextran or trehalose and dextran; sugar and PEG, for example PEG8000 and saccharides; sugar and PVP for example sucrose and PVP; sugar and amino acids, for example glycine and sucrose; two sugars together, for xample sucrose and glucose or sucrose and raffinose; sucrose and polyols, for example sucrose and sorbitol or sucrose and mannitol; polyols and amino acids, such as mannitol and glycine.

Surfactants such as polysorbate or Tween® may be added to any combination of excipients.

In order to form an immunogenic composition which can be used for vaccination, the lyophilised composition containing the antigen and the TLR9 ligand is reconstituted with a pharmaceutically acceptable diluent. It is a preferred aspect of the invention that such diluent should be a particulate diluent, for example a solution of metal salt particles, or lipsomes, or an oil in water emulsion.

In one embodiment, the diluent contains further immunostimulants. This means that the final reconstituted immunogenic composition will contain other immunostimulants in addition to the TLR9 ligand found in the lyophilised composition.

There are a number of known immunostimulants which are known to be adjuvants either alone or in combination. The innate or natural immune system recognises a wide spectrum of pathogens without a need for prior exposure. The main cells responsible for innate immunity, monocytes/macrophages and neutrophils, phagocytose microbial pathogens and trigger the innate, inflammatory, and specific immune responses.

Lipopolysaccharides (LPS) are the major surface molecule of, and occur exclusively in, the external leaflet of the outer membrane of gram-negative bacteria. LPS have been shown to be TLR4 ligands. LPS impede destruction of bacteria by serum complements and phagocytic cells, and are involved in adherence for colonisation. LPS are a group of structurally related complex molecules of approximately 10,000 Daltons in size and consist of three covalently linked regions:

(i) an *O*-specific polysaccharide chain (*O*-antigen) at the outer region
(ii) a core oligosaccharide central region
(iii) lipid A - the innermost region which serves as the hydrophobic anchor, it comprises glucosamine disaccharide units which carry long chain fatty acids.

The biological activities of LPS, such as lethal toxicity, pyrogenicity and adjuvanticity, have been shown to be related to the lipid A moiety. In contrast, immunogenicity is associated with the *O*-specific polysaccharide component (*O*-antigen). Both LPS and lipid A have long been known for their strong adjuvant effects, but the high toxicity of these molecules has precluded their use in vaccine formulations. Significant effort has therefore been made towards reducing the toxicity of LPS or lipid A while maintaining their adjuvanticity.

The *Salmonella minnesota* mutant R595 was isolated in 1966 from a culture of the parent (*smooth*) strain (Luderitz *et al.* 1966 *Ann. N. Y. Acad. Sci.* 133:349-374). The colonies selected were screened for their susceptibility to lysis by a panel of phages, and only those colonies that displayed a narrow range of sensitivity (susceptible to one or two phages only) were selected for further study. This effort led to the isolation of a *deep rough* mutant strain which is defective in LPS biosynthesis and referred to as *S. minnesota* R595.

In comparison to other LPS, those produced by the mutant *S. minnesota* R595 have a relatively simple structure.

(i) they contain no *O*-specific region - a characteristic which is responsible for the shift from the wild type smooth phenotype to the mutant rough phenotype and results in a loss of virulence
(ii) the core region is very short - this characteristic increases the strain susceptibility to a variety of chemicals
(iii) the lipid A moiety is highly acylated with up to 7 fatty acids.

4'-monophosporyl lipid A (MPL), which may be obtained by the acid hydrolysis of LPS extracted from a deep rough mutant strain of gram-negative bacteria, retains the adjuvant properties of LPS while demonstrating a toxicity which is reduced by a factor of more than 1000 (as measured by lethal dose in chick embryo eggs) (Johnson *et al.* 1987 *Rev. Infect. Dis.* 9 Suppl:S512-S516). LPS is typically refluxed in mineral acid solutions of moderate strength (e.g. 0.1 M HCl) for a period of approximately 30 minutes. This process results in dephosphorylation at the 1 position, and decarbohydration at the 6' position, yielding MPL.

3-*O*-deacylated monophosphoryl lipid A (3D-MPL), which may be obtained by mild alkaline hydrolysis of MPL, has a further reduced toxicity while again maintaining adjuvanticity, see US4,912,094 (Ribi Immunochemicals). Alkaline hydrolysis is typically performed in organic solvent, such as a mixture of chloroform/methanol, by saturation with an aqueous solution of weak base, such as 0.5 M sodium carbonate at pH 10.5.

Further information on the preparation of 3D-MPL is available in, for example, US4,912,094 and WO02/078637 (Corixa Corporation).

Some molecules which are not TLR ligands have been shown to have adjuvant activity. Quillaja saponins are a mixture of triterpene glycosides extracted from the bark of the tree

*Quillaja saponaria.* Crude saponins have been extensively employed as veterinary adjuvants. Quil-A is a partially purified aqueous extract of the Quillaja saponin material. QS21 is a Hplc purified non toxic fraction of Quil A and its method of its production is disclosed (as QA21) in US patent No. 5,057,540.

In one aspect of the invention, the diluent contains one further immunostimulant. In another aspect of the invention, the diluent contains more than one further immunostimulant. Such immunostimulants may be TLR4 ligands, saponins, TLR7 ligands, TLR8 ligands or TLR9 ligands. In one embodiment of the invention, the further immunostimulant is a TLR4 ligand such as 3D-MPL as described herein. In a further embodiment of the invention, the further immunostimulant is QS21 as described herein. In yet a further embodiment of the invention, the diluent contains QS21 and 3D-MPL. In one aspect of this embodiment, the diluent is an oil in water emulsion containing QS21 and 3D-MPL. In another aspect of this embodiment, the diluent is a solution of liposomes containing QS21 and 3D-MPL.

The invention will now be described further by way of reference to the following, non-limiting examples.

Examples

**Example 1: Freeze drying of a CpG oligonucleotide and CPC-P501S as antigen**

The antigen used was CPC – P501S. This antigen is shown in figure 1 diagrammatically, in which the section showing TM2 to TM12 represents the P501S antigen; the oval shapes on the left hand side represent the CPC fusion partners and the His tail is shown on the right hand side.

The antigen was produced with a His tag as shown in *S. cerevisiae* and then made to a concentration of 700µg/ml using a buffer of Tris (5mM pH7.5) and Tween80 (0.3%).

To prepare the final bulk, sucrose (35%) was added to water for injection to reach a final concentration of 6.3%. Tris (1M pH8.8) was then added, followed by Tween 80 (25%) to reach a final concentration of 0.2%. This mixture was magnetically stirred for 5 minutes at room temperature. CPC-P501S was added and the mixture was magnetically stirred for 4 minutes at room temperature. A CpG oligo of SEQ ID No:4 was then added, and the resulting mixture magnetically stirred for 15 minutes at room temperature to give the final bulk. The composition was analysed as follows:

| | Final Bulk (500 µl) | Final container (500 µl) Human dose |
|---|---|---|
| Cakes | | *After reconstitution with 625 µl AS01B* |
| CPC-P501 | 125 µg | 100 µg |
| CpG | 625 µg | 500 µg |
| Tris | 50 mM | 40 mM |
| Tween 80 | 0.50 % | 0.40 % |
| Saccharose | 6.3 % | 5.0 % |
| pH | 9.1 +/- 0.1 | 7.4 +/- 0.1 |

| | Final Bulk (500 µl) | Final container (500 µl) Human dose |
|---|---|---|
| Cakes | | *After reconstitution with 625 µl AS01B* |
| CPC-P501 | 25 µg | 20 µg |
| CpG | 625 µg | 500 µg |
| Tris | 50 mM | 40 mM |
| Tween 80 | 0.20 % | 0.16 % |
| Saccharose | 6.3 % | 5.0 % |
| pH | 9.1 +/- 0.1 | 7.4 +/- 0.1 |

0.5ml of a composition was filled into a glass vial, which was put through the lyophilisation cycle as shown in figure 2.

Cake characterisation was carried out by visual inspection and diameter measurement at T0, 1 week, 2 weeks, 3 weeks, and 4 weeks at $37^0$C, on three vials of the composition (see figure 3). Residual humidity content was measured at the same timepoints and temperature using thermogravimetry (TG) or Karl Fischer (KF). As can be seen below, the cakes were stable for up to two weeks.

Freeze-dried cake

| Stability timing | Visual aspect | Cake diameter (mm) | Moisture content (% $wH_2O$/w cake) | |
|---|---|---|---|---|
| | | | KF | TG |
| T0 | OK | 12.6 ± 0.1 | 0.3% (1.5 month at 4°C) | 0.8% (5 month at 4°C) |
| 1 week 37°C | OK | nd | 0.59% | nd |
| 2 week 37°C | Retraction + | 9.8 ± 0.8 | nd | 1.4% |
| 3 week 37°C | Retraction ++ | 7.7 ± 1.0 | nd | 1.2% |
| 4 week 37°C | Retraction ++ | 8.7 ± 1.5 | Not measurable | 1.3% |

KF: Karl Fischer method
TG: Thermogravimetry method
nd: not done
OK: neither aggregation nor degradation
Specs: 3% (Thermogravimetry)

The humidity in a final container stored at 37°C (to accelerate stability analysis) increases during time. After 1 month at 37°C, cakes contain 1.3% H2O and are retracted. In this experiment, the increase in humidity is due to the fact that hygroscopic powder absorbs water from the stoppers. Replacing the stoppers with new types of stoppers can help prevent this retraction.

The cakes were then reconstituted either with water for injection, or with the following carrier liquids: Adjuvant system A (a liposomal adjuvant prepared as set out in WO2005/112991), Adjuvant system E (an oil in water emulsion adjuvant prepared as set out in WO2005/112991) or adjuvant system F (an oil in water emulsion adjuvant prepared as set out in WO2005/112991).

No protein aggregation or degradation was seen with water for injection, adjuvant system E or adjuvant system F. Some aggregation and degradation was seen with adjuvant system A. It was concluded that this was due to the decrease of the pH below the isoelectric point of CPC-P501S. An increase in the concentration of the Tris excipient to 50mM solved the problem and no aggregation was then seen with adjuvant system A. It was also found that the presence of CpG in the lyo cake (i.e. co-lyophilisation of antigen

and CpG oligonucleotide) helped prevent aggregation of the antigen when reconstituted with adjuvant system A. A comparison of reconstitution of lyo cakes with and without CpG using adjuvant system A showed that there was reduced aggregation following co-lyophilisation (data not shown)

The impact of the excipients of the size of the liposomes in adjuvant system A was also studied, and it was found that there was no difference in size between liposomes found in a vial of adjuvant system A alone and liposomes found in a vial of adjuvant system A after reconstitution of a lyo-cake containing antigen, CpG, Tris and Tween. Therefore we can conclude that the components of the lyo-cake do not affect the adjuvant system (figure 4)

Finally, the antigenicity of the formulation was studied, and it was found that in terms of lymphoproliferation and intracellular cytokine (IFNγ) production, there was no difference between a liquid versus a lyo formulation of CPC-P501S (data not shown). Therefore we can conclude that the immunogenicity of the antigen is unaffected by co-lyophilisation with CpG.

**Example 2: Freeze drying of a CpG oligonucleotide and Mage-3 as antigen**

The antigen used was a portion of the protein D protein linked to MAGE-3, which in turn was linked to a His tail for ease of purification PD-Mage3-His (see Figure 5: SEQ ID NO: 13).

The purified bulk antigen was produced with a His tag in E. coli and then made to a concentration of 750 µg/ml using a buffer of $NaH_2PO_4.2H_2O/K_2HPO_4.2H_2O$ (2mM) and Tween80 at approximately 0.2%v/v (theoreitical) pH7.5.

To prepare the final bulk, sucrose (30%) was added to water for injection to give a final concentration of 3.15%. $NaH_2PO_4.2H_2O/K_2HPO_4.2H_2O$ (100mM pH7.5) was then added to give a final $PO_4$ concentration of 5mM taking into account the phosphate found in the antigen buffer. Tween 80 (3%) was also added to give a final concentration of 0.15%, taking into account the Tween found in the antigen buffer. This mixture was magnetically stirred for between 5 and 15 minutes at room temperature. PD-Mage3-His was added (750µg/ml) and the mixture was magnetically stirred for 5 - 15 minutes at room temperature. A CpG oligo of Seq ID No:4 was then added, and the resulting mixture magnetically stirred for 15 minutes (+/- 5 minutes) at room temperature to give the final bulk. The pH was adjusted to pH7.5 +/- 0.1 with NaOH 0.05M or 0.5M, or HCl 0.03M or 0.3M.

The composition was analysed as follows:

| No. | Ingredients | | | Before freeze-drying | | | Per HD (after reconstit. With 0.625 ml of diluent) | |
|---|---|---|---|---|---|---|---|---|
| | *Name* | *Component* | *Src* | *CC* | *Weight (in 0.5ml)* | | *Concentrat* | *Weight (in 0.5ml)* |
| 1 | PD-Mage3-His | NaH2PO4.2H2O-K2HPO4.3H20 2mM/Tween 80 ~0.2%v/v theo pH7.5 | | 750µg/ml | 375 µg | | 600 µg/ml | 300 µg |
| 2 | CpG | | | 1250 µg/ml | 625 µg | | 1000 µg/ml | 500 µg |
| 3 | Saccharose | | | 3.15% w/v | 15.75mg | | 2.52% w/v | 12.6mg |
| 4 | Tween 80 | | 1 | 0.15% w/v | | | 0.12% w/v | |
| 5 | PO4 | | 1 | 5mM | | | 4mM | |
| 6 | WFI | | | | | ad 0.5ml | | |
| 7 | pH | | | | | 7.5±0.1 | | |

0.5ml of this composition was filled into a glass vial, which was put through the lyophilisation cycle shown in Figure 6.

The Impact of excipients and freeze-drying cycle on cake composition was analysed after between 7 to 9 days of cake storage at 37°C .

**cake aspect and residual humidity**

| **Cake aspect** | No collapse (T0) | No retraction **(T7d 37°C)** |
|---|---|---|
| **Residual humidity** | - | 0.59% **(T8d 37°C)** |

It can be seen that the cakes do not present any collapse at 7 days and do not change through 8 days of stress stability.

Residual humidity of cakes stored for between 7 to 9 days at 37°C stays below the specification of 3%.

There was no evolution in the diameter following storage for between 7 to 9 days at 37°C.

The cakes were then reconstituted with Adjuvant system A (a liposomal adjuvant prepared as set out in WO2005/112991). No protein aggregation or degradation was seen, thereby confirming that the antigen can be co-lyophilised with CpG without affecting its ability to be reconstituted.

The antigenicity of the formulation was studied. It was found that, following reconstitution in Adjuvant system A, there was a decrease in antigenicity with time, after 24 hours. It is thought that this is due to the acidic pH (6.2+/- 0.1) found following reconstitution. This was confirmed when it was found that the antigenicity fall could be decreased by increasing the pH. However there was still some decrease in antigenicity over time. Therefore the formulations were tested to see if this decrease had an effect on the *in-vivo* potency test. Dilutions of 3/10, 1/10 and 1/30$^{th}$ of a human dose were given to groups of mice, 10 mice per group as shown in Figure 7. Mice were bled at day 28.

T0, 4h and 24h are the times following reconstitution of the cake with adjuvant system A. As can be seen in Figure 7, there was no effect on potency.

**Example 3: Impact of CpG on antigen solubility following reconstitution.**

1. WT1 is a protein originally found to be overexpressed in paediatric kidney cancer, Wilm's Tumor. The candidate antigen used in the present case uses nearly the full length protein as antigen. The WT1-A10 protein is a 292 AA recombinant fusion protein expressed in E. coli consisting of a 12mer truncated tat sequence (leader sequence) and amino acids number 2 – 281 of the WT1 sequence. After lyophilisation alone, this antigen precipates if reconstituted with adjuvant system A due to its isoelectric point (5.85 to 7.5) which is close to the pH of adjuvant system A (6.1) and the presence of sodium chloride in adjuvant system A.

Two formulations of WT1-A10 were prepared. The reconstituted dose contained 400µg/ml of WT1-A10 antigen, 10% sucrose, 100mM Tris, and 0.2% Tween 80, plus or minus 840 µg/ml CpG.

Both formulations were reconstituted with 500µl of adjuvant system A. The resulting liquid was centrifuged and a Western blot performed on the non-centrifuged liquid (NC), the supernatant (SN) and the pellet (P). The results are shown in Figure 8.

As can be seen in Figure 8, in the presence of CpG, the solubility of the antigen after reconstitution is improved as evidenced by the lack of antigen in the precipitate pellet. Precipitated antigen can be seen in the pellet of the reconstituted lyophilised composition where the lyo cake did not contain CpG. This is evidence that, in the case of a non-positively charged antigen, the co-lyophilisation of CpG improves the solubility of the antigen on reconstitution.

2. PRAME

Two formulations of PRAME were prepared. The reconstituted dose contained 1000µg/ml of PRAME antigen, 3.15% sucrose, 5mM Borate, 150nM Sodium Chloride, plus or minus 840 µg/ml CpG. Both formulations were reconstituted with 500µl of adjuvant system A. The resulting liquid was centrifuged and a Western blot performed on the non-centrifuged liquid (NC), the supernatant (SN) and the pellet (P). The results are shown in Figure 9, where NC = non-centrifuged, SN = supernatant and P = pellet

As can be seen in Figure 9, in the presence of CpG, the solubility of the antigen after reconstitution is improved as evidenced by the lack of antigen in the precipitate pellet. Precipitated antigen can be seen in the pellet of the reconstituted lyophilised composition where the lyo cake did not contain CpG. This is further evidence that, in the case of a non-positively charged antigen, the co-lyophilisation of CpG improves the solubility of the antigen on reconstitution.

SEQ ID NO:1
TCC ATG ACG TTC CTG ACG TT

SEQ ID NO:2
TCT CCC AGC GTG CGC CAT

SEQ ID NO:3
ACC GAT GAC GTC GCC GGT GAC GGC ACC ACG

SEQ ID NO:4
TCG TCG TTT TGT CGT TTT GTC GTT

SEQ ID NO:5
TCC ATG ACG TTC CTG ATG CT

SEQ ID NO:6

```
MKVKETRKNY QHLWRWGTML LGMLMICSAA EQLWVTVYYG VPVWKEATTT    50
LFCASDAKAY DTEVHNVWAT HACVPTDPNP QEVVLGNVTE YFNMWKNNMV   100
DQMHEDIISL WDQSLKPCVK LTPLCVTLDC DDVNTTNSTT TTSNGWTGEI   150
RKGEIKNCSF NITTSIRDKV QKEYALFYNL DVVPIDDDNA TTKNKTTRNF   200
RLIHCNSSVM TQACPKVSFE PIPIHYCAPA GFAILKCNNK TFDGKGLCTN   250
VSTVQCTHGI RPVVSTQLLL NGSLAEEEVV IRSDNFMDNT KTIIVQLNES   300
VAINCTRPNN NTRKGIHIGP GRAFYAARKI IGDIRQAHCN LSRAQWNNTL   350
KQIVIKLREH FGNKTIKFNQ SSGGDPEIVR HSFNCGGEFF YCDTTQLFNS   400
TWNGTEGNNT EGNSTITLPC RIKQIINMWQ EVGKAMYAPP IGGQIRCSSN   450
ITGLLLTRDG GTEGNGTENE TEIFRPGGGD MRDNWRSELY KYKVVKVEPL   500
GVAPTRAKRR VVQR                                          514
```

SEQ ID NO:7

```
   1  MRVMEIQRNC QHLLRWGIMI LGMIIICSTA DNLWVTVYYG VPVWRDAETT
  51  LFCASDAKAY STEKHNVWAT HACVPTDPNP QEIPLDNVTE EFNMWKNNMV
 101  DQMHEDIISL WDQSLKPCVQ LTPLCVTLNC SNARVNATFN STEDREGMKN
 151  CSFNMTTELR DKKQQVYSLF YRLDIEKINS SNNNSEYRLV NCNTSAITQA
 201  CPKVTFEPIP IHYCAPAGFA ILKCNDTEFN GTGPCKNVST VQCTHGIKPV
 251  VSTQLLLNGS LAEREVRIRS ENIANNAKNI IVQFASPVKI NCIRPNNNTR
 301  KSYRIGPGQT FYATDIVGDI RQAHCNVSRT DWNNTLRLVA NQLRKYFSNK
 351  TIIFTNSSGG DLEITTHSFN CGGEFFYCNT SGLFNSTWTT NNMQESNDTS
 401  NGTITLPCRI KQIIRMWQRV GQAMYAPPIE GVIRCESNIT GLILTRDGGN
 451  NNSANETFRP GGGDIRDNWR SELYKYKVVK IEPLGVAPTR AKRRVVEREK
 501  RAVGIGAVFL GFLGAAGSTM GAASITLTVQ ARQLLSGIVQ QQSNLLRAIE
 551  AQQQLLKLTV WGIKQLQARV LAVERYLRDQ QLLGIWGCSG KLICTTNVPW
 601  NSSWSNKSYD DIWQNMTWLQ WDKEISNYTD IIYSLIEESQ NQQEKNEQDL
 651  LALDKWANLW NWFDISKWLW YIRS
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SEQ ID NO:8

```
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  51  LETSEGCRQI LGQLQPSLQT GSEELRSLYN TVATLYCVHQ RIEIKDTKEA
 101  LDKIEEEQNK SKKKAQQAAA DTGHSNQVSQ NYPIVQNIQG QMVHQAISPR
 151  TLNAWVKVVE EKAFSPEVIP MFSALSEGAT PQDLNTMLNT VGGHQAAMQM
 201  LKETINEEAA EWDRVHPVHA GPIAPGQMRE PRGSDIAGTT STLQEQIGWM
 251  TNNPPIPVGE IYKRWIILGL NKIVRMYSPT SILDIRQGPK EPFRDYVDRF
 301  YKTLRAEQAS QEVKNWMTET LLVQNANPDC KTILKALGPA ATLEEMMTAC
 351  QGVGGPGHKA RVLMGPISPI ETVPVKLKPG MDGPKVKQWP LTEEKIKALV
 401  EICTEMEKEG KISKIGPENP YNTPVFAIKK KDSTKWRKLV DFRELNKRTQ
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 451  DFWEVQLGIP HPAGLKKKKS VTVLDVGDAY FSVPLDEDFR KYTAFTIPSI
 501  NNETPGIRYQ YNVLPQGWKG SPAIFQSSMT KILEPFRKQN PDIVIYQYMD
 551  DLYVGSDLEI GQHRTKIEEL RQHLLRWGLT TPDKKHQKEP PFLKMGYELH
 601  PDKWTVQPIV LPEKDSWTVN DIQKLVGKLN WASQIYPGIK VRQLCKLLRG
 651  TKALTEVIPL TEEAELELAE NREILKEPVH GVYYDPSKDL IAEIQKQGQG
 701  QWTYQIYQEP FKNLKTGKYA RMRGAHTNDV KQLTEAVQKI TTESIVIWGK
 751  TPKFKLPIQK ETWETWWTEY WQATWIPEWE FVNTPPLVKL WYQLEKEPIV
 801  GAETFYVDGA ANRETKLGKA GYVTNRGRQK VVTLTDTTNQ KTELQAIYLA
 851  LQDSGLEVNI VTDSQYALGI IQAQPDQSES ELVNQIIEQL IKKEKVYLAW
 901  VPAHKGIGGN EQVDKLVSAG IRKVLMVGFP VTPQVPLRPM TYKAAVDLSH
 951  FLKEKGGLEG LIHSQRRQDI LDLWIYHTQG YFPDWQNYTP GPGVRYPLTF
1001  GWCYKLVPVE PDKVEEANKG ENTSLLHPVS LHGMDDPERE VLEWRFDSRL
1051  AFHHVARELH PEYFKNC
```

SEQ ID NO:9

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ccttcagacaggatcagaagaacttagatcattatataatacagtagcaaccctctattgtgtgcatcaaagg
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agcaagcagcagctgacacaggacacagcaatcaggtcagccaaaattactaa

SEQ ID NO:10

**MVIVQNIQGQMVHQAISPRTLNAWVKVVEEKAFSPEVIPMFSALSEGATP** 50
**QDLNTMLNTVGGHQAAMQMLKETINEEAAEWDRVHPVHAGPIAPGQMREP** 100
**RGSDIAGTTSTLQEQIGWMTNNPPIPVGEIYKRWIILGLNKIVRMYSPTS** 150
**ILDIRQGPKEPFRDYVDRFYKTLRAEQASQEVKNWMTETLLVQNANPDCK** 200
**TILKALGPAATLEEMMTACQGVGGPGHKARVL**HMGPISPIETVSVKLKPG 250
MDGPKVKQWPLTEEKIKALVEICTEMEKEGKISKIGPENPYNTPVFAIKK 300
KDSTKWRKLVDFRELNKRTQDFWEVQLGIPHPAGLKKKKSVTVLDVGDAY 350
FSVPLDEDFRKYTAFTIPSINNETPGIRYQYNVLPQGWKGSPAIFQSSMT 400
KILEPFRKQNPDIVIYQYMDDLYVGSDLEIGQHRTKIEELRQHLLRWGLT 450
TPDKKHQKEPPFLKMGYELHPDKWTVQPIVLPEKDSWTVNDIQKLVGKLN 500
WASQIYPGIKVRQLCKLLRGTKALTEVIPLTEEAELELAENREILKEPVH 550
GVYYDPSKDLIAEIQKQGQGQWTYQIYQEPFKNLKTGKYARMRGAHTNDV 600
KQLTEAVQKITTESIVIWGKTPKFKLPIQKETWETWWTEYWQATWIPEWE 650
FVNTPPLVKLWYQLEKEPIVGAETFYVDGAANRETKLGKAGYVTNRGRQK 700
VVTLTDTTNQKTELQAIYLALQDSGLEVNIVTDSQYALGIIQAQPDQSES 750
ELVNQIIEQLIKKEKVYLAWVPAHKGIGGNEQVDKLVSAGIRKVLAMGGK 800
WSKSSVVGWPTVRERMRRAEPAADGVGAASRDLEKHGAITSSNTAATNAA 850
CAWLEAQEEEEVGFPVTPQVPLRPMTYKAAVDLSHFLKEKGGLEGLIHSQ 900
RRQDILDLWIYHTQGYFPDWQNYTPGPGVRYPLTFGWCYKLVPVEPDKVE 950
EANKGENTSLLHPVSLHGMDDPEREVLEWRFDSRLAFHHVARELHPEYFK 1000
NCRPMGARASVLSGGELDRWEKIRLRPGGKKKYKLKHIVWASRELERFAV 1050
NPGLLETSEGCRQILGQLQPSLQTGSEELRSLYNTVATLYCVHQRIEIKD 1100
TKEALDKIEEEQNKSKKKAQQAAADTGHSNQVSQNY 1136

SEQ ID NO:11

```
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  51  LETTEGCQQI MNQLQPAVKT GTEEIKSLFN TVATLYCVHQ RIDVKDTKEA
 101  LDKIEEIQNK SKQKTQQAAA DTGDSSKVSQ NYPIIQNAQG QMIHQNLSPR
 151  TLNAWVKVIE EKAFSPEVIP MFSALSEGAT PQDLNVMLNI VGGHQAAMQM
 201  LKDTINEEAA EWDRLHPVQA GPIPPGQIRE PRGSDIAGTT STPQEQLQWM
 251  TGNPPIPVGN IYKRWIILGL NKIVRMYSPV SILDIKQGPK EPFRDYVDRF
 301  FKALRAEQAT QDVKGWMTET LLVQNANPDC KSILKALGSG ATLEEMMTAC
 351  QGVGGPGHKA RVLAEAMSQA QQTNIMMQRG NFRGQKRIKC FNCGKEGHLA
 401  RNCRAPRKKG CWKCGKEGHQ MKDCTERQAN FLGKIWPSSK GRPGNFPQSR
 451  PEPTAPPAEL FGMGEGIASL PKQEQKDREQ VPPLVSLKSL FGNDPLSQGS
 501  PISPIETVPV TLKPGMDGPK VKQWPLTEEK IKALTEICTE MEKEGKISKI
 551  GPENPYNTPI FAIKKKDSTK WRKLVDFREL NKRTQDFWEV QLGIPHPAGL
 601  KKKKSVTVLD VGDAYFSVPL DENFRKYTAF TIPSTNNETP GVRYQYNVLP
 651  QGWKGSPAIF QSSMTKILEP FRSKNPEIII YQYMAALYVG SDLEIGQHRT
 701  KIEELRAHLL SWGFTTPDKK HQKEPPFLWM GYELHPDKWT VQPIMLPDKE
 751  SWTVNDIQKL VGKLNWASQI YAGIKVKQLC RLLRGAKALT DIVTLTEEAE
 801  LELAENREIL KDPVHGVYYD PSKDLVAEIQ KQGQDQWTYQ IYQEPFKNLK
 851  TGKYARKRSA HTNDVRQLAE VVQKVAMESI VIWGKTPKFK LPIQKETWET
 901  WWMDYWQATW IPEWEFVNTP PLVKLWYQLE KDPILGAETF YVDGAANRET
 951  KLGKAGYVTD RGRQKVVSLT ETTNQKTELH AILLALQDSG SEVNIVTDSQ
1001  YALGIIQAQP DRSESELVNQ IIEKLIGKDK IYLSWVPAHK GIGGNEQVDK
1051  LVSSGIRKVL FLDGIDKAQE DHERYHSNWR TMASDFNLPP IVAKEIVASC
1101  DKCQLKGEAM HGQVDCSPGI WQLACTHLEG KVILVAVHVA SGYIEAEVIP
1151  AETGQETAYF LLKLAGRWPV KVVHTANGSN FTSAAVKAAC WWANIQQEFG
1201  IPYNPQSQGV VASMNKELKK IIGQVRDQAE HLKTAVQMAV FIHNFKRKGG
1251  IGGYSAGERI IDIIATDIQT KELQKQITKI QNFRVYYRDS RDPIWKGPAK
1301  LLWKGEGAVV IQDNSDIKVV PRRKAKILRD YGKQMAGDDC VAGRQDEDRS
1351  MGGKWSKGSI VGWPEIRERM RRAPAAAPGV GAVSQDLDKH GAITSSNINN
1401  PSCVWLEAQE EEEVGFPVRP QVPLRPMTYK GAFDLSHFLK EKGGLDGLIY
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1451 SRKRQEILDL WVYHTQGYFP DWQNYTPGPG VRYPLTFGWC FKLVPMEPDE
1501 VEKATEGENN SLLHPICQHG MDDEEREVLI WKFDSRLALK HRAQELHPEF
1551 YKDC

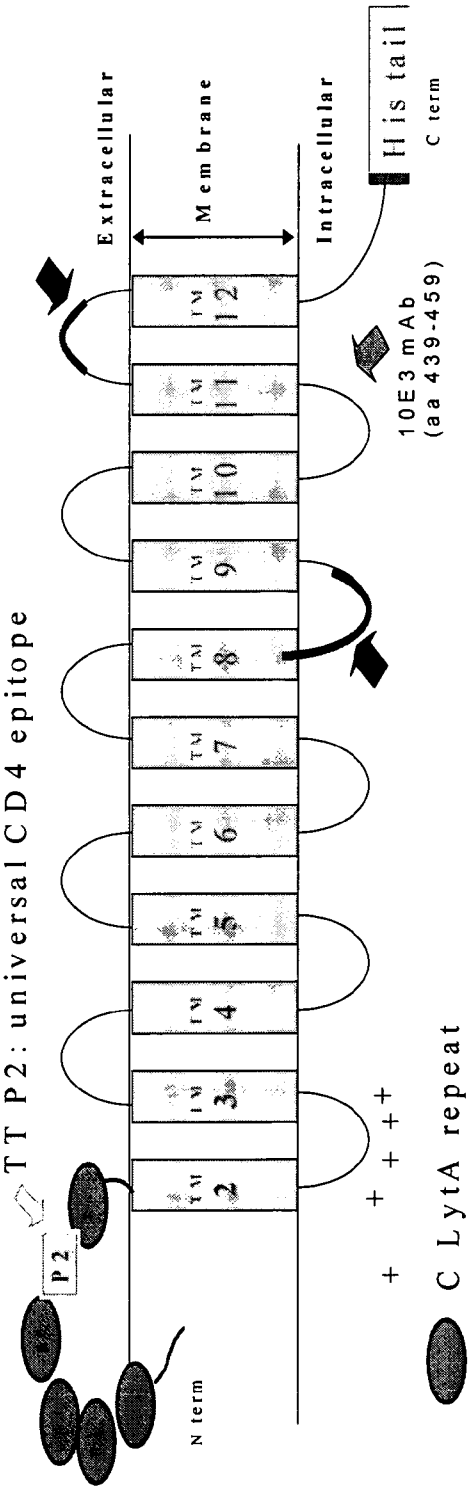
SEQ ID NO:12

Protein D_H influenzae (1) MKLKTLALSLLAAGVLAGCSSHSSNMANTQMKSDKIIIAHRGASGYLPEH
51) TLESKALAFAQQADYLEQDLAMTKDGRLVVIHDHFLDGLTDVAKKFPHRH(101) RKDGRYYVIDFTLKEIQSLEMTENFET

CLAIMS

1. A lyophilised composition comprising one or more antigens and a TLR9 agonist.
2. A composition according to claim 1 wherein said TLR9 agonist is an immunostimulatory oligonucleotide.
3. A composition according to claim 2 wherein said immunostimulatory oligonucleotide is a CpG containing oligonucleotide.
4. A composition according to claim 3 wherein said immunostimulatory oligonucleotide comprises a Purine, Purine, C, G, pyrimidine, pyrimidine sequence.
5. A composition as claimed in claims 2 to 4, wherein said immunostimulatory oligonucleotide is selected from the group comprising:
   TCC ATG ACG TTC CTG ACG TT (SEQ ID NO:1);
   TCT CCC AGC GTG CGC CAT (SEQ ID NO:2);
   ACC GAT GAC GTC GCC GGT GAC GGC ACC ACG (SEQ ID NO:3);
   TCG TCG TTT TGT CGT TTT GTC GTT (SEQ ID NO:4);
   TCC ATG ACG TTC CTG ATG CT (SEQ ID NO:5).
6. A composition according to any of claims claims 2 to 4, wherein the immunostimulatory oligonucleotide contains at least two unmethylated CG repeats being separated at least by 3 nucleotides.
7. A composition according to claim 6, wherein the immunostimulatory oligonucleotide contains at least two unmethylated CG repeats being separated by 6 nucleotides.
8. A composition according to any of claims 1 to 8 wherein said antigen(s) is not a positively charged antigen.
9. A composition according to any of claims 1 to 8 wherein one or more of said antigens is a tumour associated antigen.
10. A composition according to claim 9 wherein said antigen is selected from the group consisting of: MAGE-3, CPC-P501S, WT-1
11. A composition according to any of claims 1 to 8 wherein at least one of said antigens is an HIV-1 derived antigen.
12. A composition according to claim 11 wherein one of said antigens is F4.
13. A composition according to claim 11 or 12 wherein said antigens are F4 and gp120.
14. A method of making a lyophilised composition according to any of claims 1 to 12 comprising the steps of mixing the desired antigen and TLR9 ligand with suitable excipients, and submitting the resulting formulation to a lyophilisaton cycle.
15. A method of making an immunogenic composition comprising the steps of reconstituting the lyophilised composition of any of claims 1 to 12 with a suitable carrier.
16. A method according to claim 14 wherein said carrier is a particulate carrier selected from the group comprising mineral salts, emulsions, polymers, liposomes, ISCOMs.

17. A method according to claim 15 wherein said carrier is a liposomal solution or an oil in water emulsion.
18. A method according to any of claims 14 to 16 wherein said carrier further comprises one or more immunostimulants.
19. A method according to claim 17 wherein said said one or more immunostimulants are selected from the group consisting of TLR 4 agonists, TLR 4 antagonists, saponins, TLR7 agonists, TLR8 agonists, TLR9 agonists.
20. A method according to claim 18 wherein said TLR 4 antagonist is 3-deacylated MPL.
21. A method according to claim 18 or 19 wherein said saponin is QS21.
22. A method according to claim 17 wherein said carrier comprises two immunostimulants.
23. A method according to claim 21 wherein said immunostimulants are 3-deacylated MPL and QS21.




SUBSTITUTE SHEET (RULE 26)

Figure 2

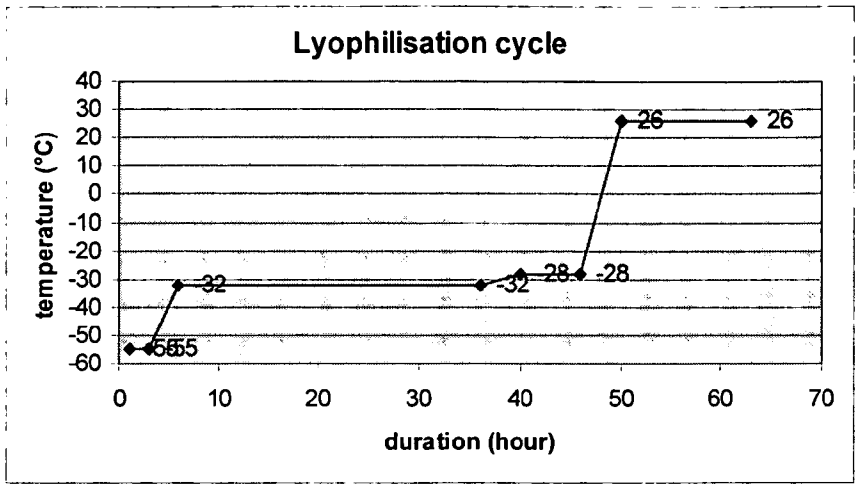

Lyophilisation cycle
temperature (°C)
40
30
20
10
0
-10
-20
-30
-40
-50
-60
0
10
20
30
40
50
60
70
duration (hour)
-5
-32
-32
-28
-28
26
26

**Figure 3**

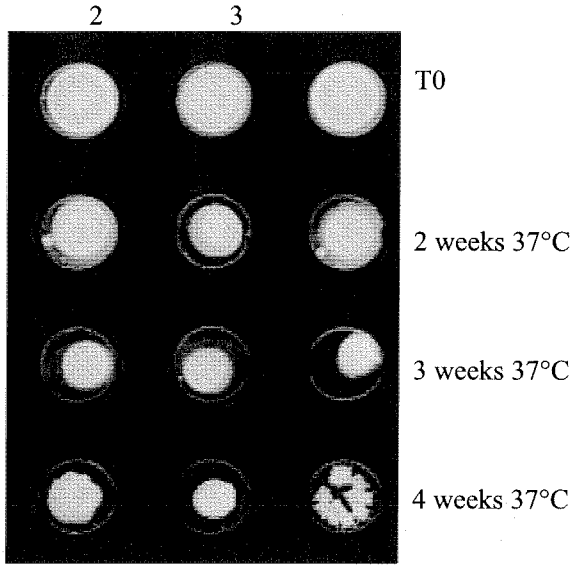


**Figure 4**

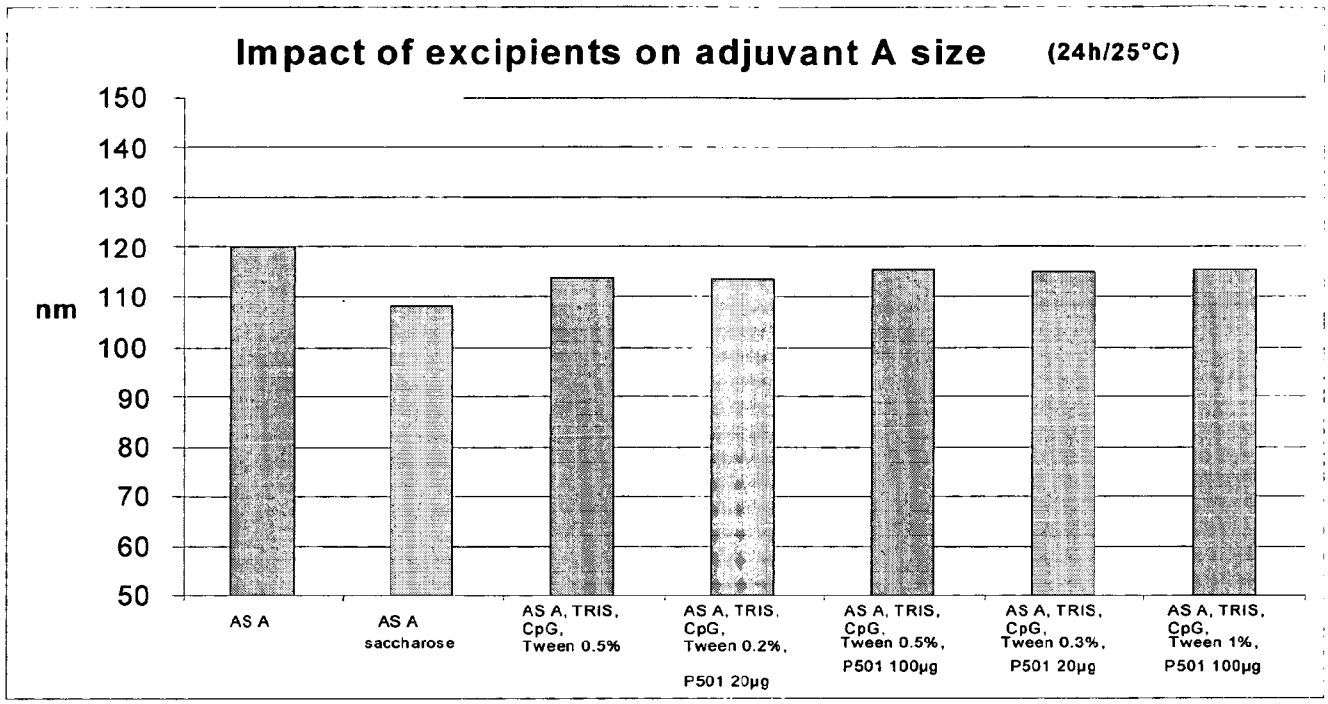
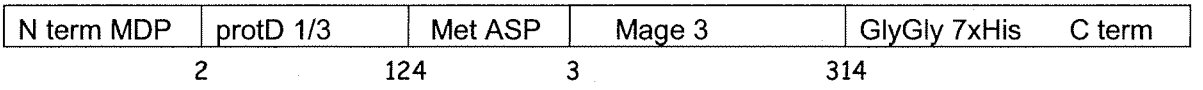

Impact of excipients on adjuvant A size (24h/25°C)
nm
150
140
130
120
110
100
90
80
70
60
50
AS A
AS A saccharose
AS A, TRIS, CpG, Tween 0.5%
AS A, TRIS, CpG, Tween 0.2%, P501 20µg
AS A, TRIS, CpG, Tween 0.5%, P501 100µg
AS A, TRIS, CpG, Tween 0.3%, P501 20µg
AS A, TRIS, CpG, Tween 1%, P501 100µg

**Figure 5**

**LipoD1/3 - MAGE3 – HIS protein :**




SEQ ID NO:13

MDPKTLALSLLAAGVLAGCSSHSSNMANTQMKSDKIIIAH 40
RGASGYLPEHTLESKALAFAQQADYLEQDLAMTKDGRLVV 80
IHDHFLDGLTDVAKKFPHRHRKDGRYYVIDFTLKEIQSLE 120
MTENFETMDLEQRSQHCKPEEGLEARGEALGLVGAQAPAT 160
EEQEAASSSSTLVEVTLGEVPAAESPDPPQSPQGASSLPT 200
TMNYPLWSQSYEDSSNQEEEGPSTFPDLESEFQAALSRKV 240
AELVHFLLLKYRAREPVTKAEMLGSVVGNWQYFFPVIFSK 280
ASSSLQLVFGIELMEVDPIGHLYIFATCLGLSYDGLLGDN 320
QIMPKAGLLIIVLAIIAREGDCAPEEKIWEELSVLEVFEG 360
REDSILGDPKKLLTQHFVQENYLEYRQVPGSDPACYEFLW 400
GPRALVETSYVKVLHHMVKISGGPHISYPPLHEWVLREGE 440
EGGHHHHHHH. 451

RED = signal sequence 15aa
Blue = first 109 amino acids of Protein D
Pink = unrelated amino acids

* (MDP first aa of Influenza)
* (Met-Asp at aa 128-129 to create a cloning site))
* (Gly-Gly at 442-443).

Green = fragment of MAGE3; amino acids 3-314 of MAGE3 (312 aas total)
Orange = 7 his tail

**Figure 6**

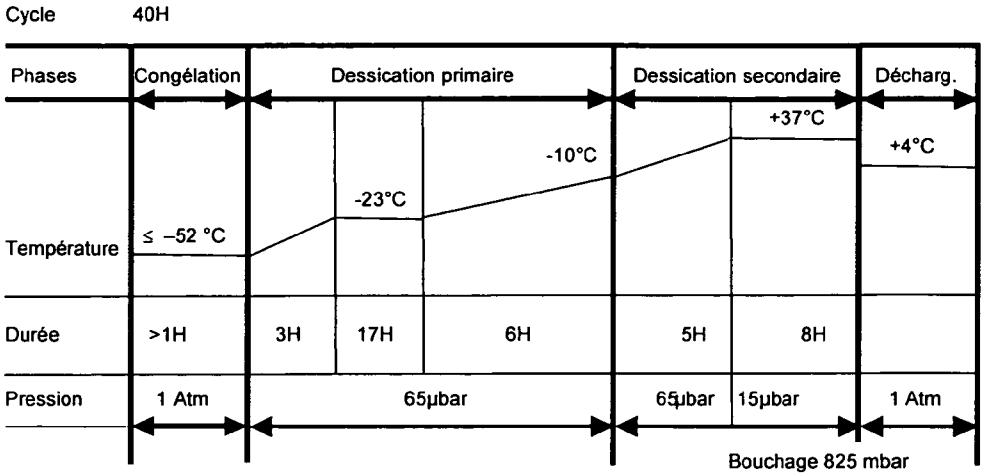

Cycle 40H
Phases
Congélation
Dessication primaire
Dessication secondaire
Décharg.
Température
≤ −52 °C
-23°C
-10°C
+37°C
+4°C
Durée
>1H
3H
17H
6H
5H
8H
Pression
1 Atm
65µbar
65µbar
15µbar
1 Atm
Bouchage 825 mbar

Figure 7

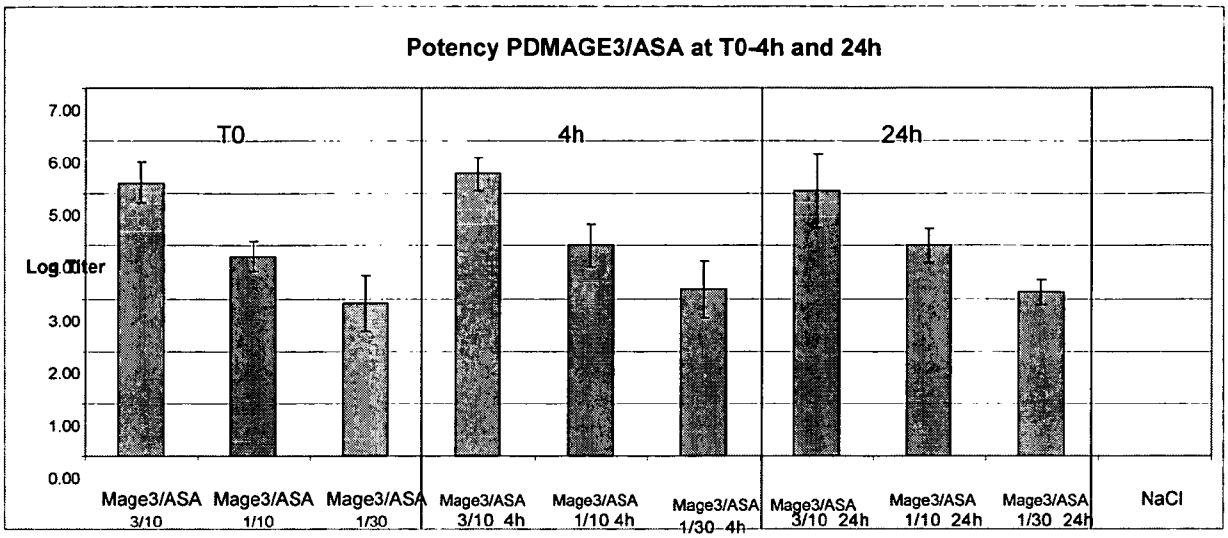

Potency PDMAGE3/ASA at T0-4h and 24h
T0
4h
24h
7.00
6.00
5.00
3.00
2.00
1.00
0.00
Log Titer
Mage3/ASA 3/10
Mage3/ASA 1/10
Mage3/ASA 1/30
Mage3/ASA 3/10 4h
Mage3/ASA 1/10 4h
Mage3/ASA 1/30 4h
Mage3/ASA 3/10 24h
Mage3/ASA 1/10 24h
Mage3/ASA 1/30 24h
NaCl

Figure 8

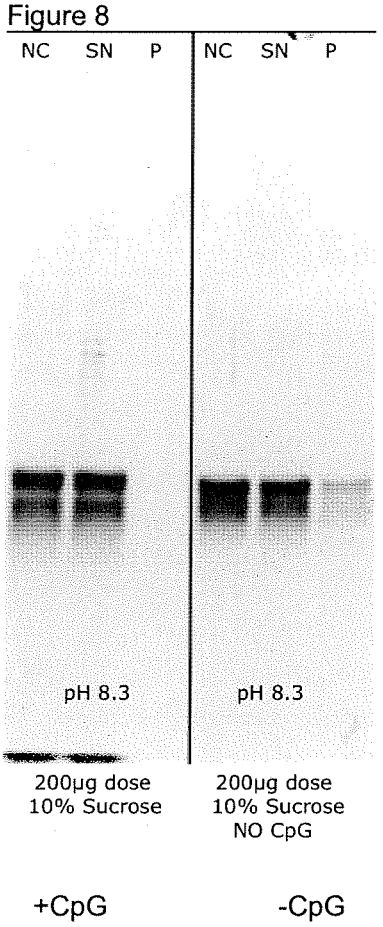
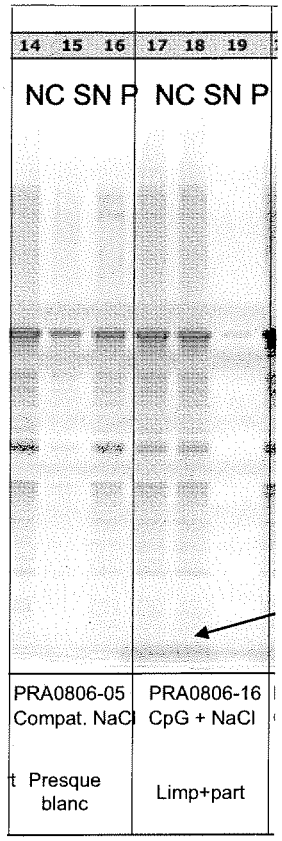

NC
SN
P
NC
SN
P
pH 8.3
pH 8.3
200µg dose
10% Sucrose
200µg dose
10% Sucrose
NO CpG
+CpG
-CpG

Figure 9




**- CpG + CpG**

# INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2008/056305

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K39/00 C07K14/47

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 C07K

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 practical, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | EP 1 547 581 A (VECTRON THERAPEUTICS AG [DE]) 29 June 2005 (2005-06-29) the whole document<br>---<br>-/-- | 1-23 |

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

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| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 3 September 2008 | 12/09/2008 |

| Name and mailing address of the ISA/ | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL – 2280 HV Rijswijk<br>Tel. (+31–70) 340–2040, Tx. 31 651 epo nl,<br>Fax: (+31–70) 340–3016 | Gruber, Andreas |

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
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| A | WO 2005/105139 A (GLAXOSMITHKLINE BIOLOG SA [BE]; BRUCK CLAUDINE ELVIRE MARIE [US]; BRIC) 10 November 2005 (2005-11-10) the whole document | 1-23 |
| A | BOON T ET AL: "Monitoring CTL responses in melanoma patients vaccinated with MAGE antigenic peptides" EUROPEAN JOURNAL OF CANCER, PERGAMON PRESS, OXFORD, GB, vol. 37, April 2001 (2001-04), page S11, XP004477279 ISSN: 0959-8049 the whole document | 1-23 |
| A | BAREN VAN N ET AL: "TUMORAL AND IMMUNOLOGIC RESPONSE AFTER VACCINATION OF MELANOMA PATIENTS WITH AN ALVAC VIRUS ENCODING MAGE ANTIGENS RECOGNIZED BY T CELLS" JOURNAL OF CLINICAL ONCOLOGY, GRUNE AND STRATTON, NEW YORK, NY, US, vol. 23, no. 35, 10 December 2005 (2005-12-10), pages 9008-9021, XP009075846 ISSN: 0732-183X the whole document | 1-23 |

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | COULIE P G ET AL: "A monoclonal cytolytic T-lymphocyte response observed in a melanoma patient vaccinated with a tumor-specific antigenic peptide encoded by gene MAGE-3." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA 28 AUG 2001, vol. 98, no. 18, 28 August 2001 (2001-08-28), pages 10290-10295, XP002447303 ISSN: 0027-8424 the whole document | 1-23 |
| A | TOUNGOUZ M ET AL: "Transient expansion of peptide-specific lymphocytes producing IFN-gamma after vaccination with dendritic cells pulsed with MAGE peptides in patients with mage-A1/A3-positive tumors." JOURNAL OF LEUKOCYTE BIOLOGY JUN 2001, vol. 69, no. 6, June 2001 (2001-06), pages 937-943, XP002447304 ISSN: 0741-5400 the whole document | 1-23 |
| A | MARCHAND M ET AL: "Tumor regressions observed in patients with metastatic melanoma treated with an antigenic peptide encoded by gene MAGE-3 and presented by HLA-A1" INTERNATIONAL JOURNAL OF CANCER, NEW YORK, NY, US, vol. 80, no. 2, 18 January 1999 (1999-01-18), pages 219-230, XP002305439 ISSN: 0020-7136 the whole document | 1-23 |
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Information on patent family members

International application No
PCT/EP2008/056305

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
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| | | CA 2544895 A1 | 14-07-2005 |
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| | | JP 2007515452 T | 14-06-2007 |
| | | US 2007148220 A1 | 28-06-2007 |
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| | | CN 1980691 A | 13-06-2007 |
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