Vaccines for Malaria

The present invention relates to a novel lipoprotein particle, methods for preparing and purifying the same, its use in medicine, particularly in the prevention of malarial infections, compositions/vaccines containing the protein or antibodies against the protein particle such as monoclonal or polyclonal antibodies and use of the same, particularly in therapy.

Malaria, is one of the world's major health problems with more than 2 to 4 million people dying from the disease each year. One of the most prevalent forms of the disease is caused by the protozoan parasite *P. vivax,* which is found in tropical and sub-tropical regions. Interestingly the parasite can complete its mosquito cycle at temperatures as low as 15 degrees Celsius, which has allowed the disease to spread in temperate climates.

One of the most acute forms of the disease is caused by the protozoan parasite, *Plasmodium falciparum* (*P. falciparum*) which is responsible for most of the mortality attributable to malaria.

The life cycle of *Plasmodium* is complex, requiring two hosts, man and mosquito for completion. The infection of man is initiated by the introduction of sporozoites in the saliva of an infected mosquito. The sporozoites migrate to the liver and there infect hepatocytes where they differentiate, via the exoerythrocytic intracellular stage, into the merozoite stage which infects red blood cells (RBC) to initiate cyclical replication in the asexual blood stage. The cycle is completed by the differentiation of a number of merozoites in the RBC into sexual stage gametocytes, which are ingested by the mosquito, where they develop through a series of stages in the midgut to produce sporozoites which migrate to the salivary gland.

Due to the fact that the disease caused by *P. vivax* is rarely lethal, efforts to prevent and treat malaria have been focused on the more deadly form of the disease caused by *Plasmodium falciparum (P. falciparum).*

Although the disease caused by *P. vivax* does not usually result in death of the patient, due to the volume of cases, which seems to be increasing, the significant impact on the quality of life of the patient, the increasing reports of the severe incidences of the disease resulting in anemia and death, and the economic impact, an effective vaccination for the disease is still required. Furthermore, a single vaccine able to provide protection against both causes of the disease would be advantageous.

A feature of the *P. vivax* is that some strains are capable of causing delayed infection by remaining latent in the liver before emerging into the peripheral circulation to manifest clinical symptoms. Thus individuals, for example when traveling through an infected area, may be infected and yet may not exhibit symptoms for several months. This has the potential to cause the spread of the disease and for this reason persons

traveling to infected areas are not allowed to donate blood for transfusion for a defined period of time after traveling to the infected region.

P. vivax malaria infection remains latent within the liver while the parasite is undergoing pre-erthrocytic shizogony. If the parasite is controlled at this stage, before it escapes the liver, no clinical symptoms of the disease, are observed in the patient.

The sporozoite stage of *Plasmodium* has been identified as a potential target of a malaria vaccine. Vaccination with deactivated (irradiated) sporozoite has been shown to induced protection against experimental human malaria (Am. J, Trop. Med. Hyg 24: 297-402, 1975). However, it is has not been possible practically and logistically to manufacture a vaccine for malaria for the general population based on this methodology, employing irradiated sporozoites.

The major surface protein of the sporozoite is known as circumsporozoite protein (CS protein). It is thought to be involved in the motility and invasion of the sporozoite during its passage from the initial site of inoculation by the mosquito into the circulation, where it migrates to the liver.

The CS protein of *Plasmodia* species is characterized by a central repetitive domain (repeat region) flanked by non-repetitive amino (N-terminus) and carboxy (C-terminus) fragments. The central domain of *P. vivax* is composed of several blocks of a repeat unit, generally of nine tandem amino acids.

In certain Asian strains, after the central repeat region, an additional sequence of approximately 12 amino acids is present (see SEQ ID No 11). The function of the latter is not known. However, it is hypothesized, by some, that said amino acids may be linked to the delayed onset of clinical symptoms of the disease, although this has not been investigated. It is thought that the N-terminus is characterised by a sequence of 5 amino acids known as region I (see SEQ ID No 1). It is also thought that the C-terminus is characterised by comprising a sequence of 18 amino acids known as region II. The latter contains a cell-adhesive motif, which is highly conserved among all malaria CS protein (see SEQ ID No. 2).

Several groups have proposed subunit vaccines based on the circumsporozoite protein. Two of these vaccines have undergone clinical testing; one is a synthetic peptide, the other is a recombinant protein (Ballou *et al* Lancet: i 1277 (1987) and Herrington *et al* Nature 328:257 (1987)). These vaccines were successful in stimulating an anti-sporozoite response. Nonetheless, the magnitude of the response was disappointing, with some vaccinees not making a response at all. Furthermore, the absence of "boosting" of antibody levels after subsequent injections and results of *in vitro* lymphocyte proliferation assays suggested that T-cells of most of these volunteers did not recognise the immuno-dominant repeat. Nonetheless, one volunteer vaccinated in each study did not develop parasitemia.

WO 93/10152 and WO 98/05355 describe a vaccine derived from the CS protein of *P. falciparum* and it seems that there has been some progress made towards the vaccination against *P. falciparum* using the approach described therein, see also Heppner et al. 2005, Vaccine 23, 2243-50.

The CS protein in *P. falciparum* has a central repeat region that is conserved. In contrast at least two forms (designated VK210 or type I and VK247 or type II) of the CS protein for *P. vivax* are known. This renders it more difficult to identify a construct of the CS protein with all the desired properties such as immogenicity, which provides general protection against *P. vivax* regardless of the specific type of CS protein because antibodies directed the central repeating region of type I do not necessarily recognize epitopes on the corresponding region of type II and *vice versa.*

A recombinant *P. vivax* CS protein was expressed and tested as a vaccine in the 1980-1990's with limited success (Collins *et al.*, 1989. Am. J. Trop. Med. Hyg. 40, 455-64). Some work has been done to develop a vaccine based on Multiple Antigen Peptides (MAP) employing one or more epitopes that are cross-linked (Nardelli and Tam, 1995, Pharm. Biotechnol. 6, 803-19).

The present invention provides an antigenic particle for use in malaria vaccines, which is believed to produce a humoral response and also a cellular immune response. The antigenic particle is believed to induce the production of antibodies against the CS protein of *P. falciparum* and *P. vivax*, type I and type II. The antigen may also induce T helper cells, for example Th1 and/or Th2 cells.

Accordingly, the present invention provides an immunogenic protein particle comprising the following monomers:

a. a fusion protein comprising sequences derived from a CS protein of *P. vivax* and the S antigen of Hepatitis B (CSV-S), and
b. a fusion protein comprising sequences derived from CS protein of *P. falciparum* and S antigen of Hepatitis B (RTS), and optionally
c. S antigen derived from Hepatitis B virus.

Sequence Listing

SEQ. ID. No. 1	Region I in the N-terminus
SEQ. ID. No. 2	Region II in the C-terminus
SEQ. ID. No. 3-9	Various repeat units of type I CS protein
SEQ. ID. No. 10	Major repeat unit from type II CS protein
SEQ. ID. No. 11	Additional amino acids found in Asian strains
SEQ. ID. No. 12	Nucleotide sequence of the hybrid protein CSV (optimized for expression in E Coli)
SEQ. ID. No. 13	Amino acid sequence of the hybrid protein CSV
SEQ. ID. No. 14	Minor repeat unit from type II CS protein
SEQ. ID. No. 15	Nucleotide sequence for the hybrid protein CSV (optimized for expression in yeast)
SEQ. ID. No. 16	Nucleotide sequence for the hybrid fusion protein CSV-S

SEQ. ID. No. 17 Amino acid sequence for the hybrid fusion protein CSV-S

SEQ. ID No. 18 Nucleotide Sequence for an RTS expression cassette and predicted RTS,S protein.

Figures

Fig 1 Plasmid map for pRIT15546 is a yeast episomal vector.

Fig 2 Plasmid map of pGF1-S2 a plasmid prepared by GSK employed in "fusing" the desired antigen with the S antigen from Hepatitis B. Cloning heterologous DNA sequences between SmaI sites (after excision of the 12bp SmaI DNA fragment) creates in-frame fusion with the S gene.

Fig 3 Plasmid map of pRIT15582
Digestion with *XhoI* liberates a 8.5 kb linear DNA fragment carrying the CSV-S expression cassette plus the LEU2 selective marker, being used for insertion into the yeast chromosome.

Fig 4 Restriction map of the linear XhoI fragment used to integrate CSV-S cassette

Fig 5 Western blot of recombinant proteins expressed in strain Y1835.
Panel A : WB revealed with anti-S antibody
Samples loaded (100µg total prortein/well):
1: Y1631 (RTS,S producing strain, as comparison)
2 : Y1835
3 : Y1835
4 : Y1834
Panel B : WB revealed with anti-CSV antibody
Samples loaded (100µg total protein/well):
1: Y1631 (RTS,S producing strain, as comparison)
2 : Y1295
3 : Y1835
4 : Y1834
5: nr (another construct CSVS)
6 : nr (another construct –S antigen only)

Fig 6 Electron micrograph of CSV-S,S mixed particles produced in strain Y1835
CSV-S,S particles were purified from soluble cell extracts (based on RTS,S purification process) and submitted to electron microscopy analysis. Particles were visualized after negative staining with phosphotungstic acid. The scale is equivalent to 100nm.

Fig 7 Western blot of recombinant proteins expressed in strain Y1845.
WB revealed with anti-S antibody
Quantity of total protein loaded is in brackets
1: Y1835 (100µg)

2: Y1631 (100μg - RTS,S producing strain, as comparison)
3 : Y1845 (100μg)
4 : Y1845 (50μg)
5 : Y1845 (25μg)

Fig 8 CsCl density analysis of a cell-free extract prepared from strain Y1845

Thus the fusion protein CSV-S employed in the invention comprises: a portion derived from the CS protein of *P. vivax* (CSV). This CSV antigen may a native protein such as found in type I CS proteins of *P.vivax* and/or as found in type II proteins of *P.vivax*. Alternatively the CSV protein may be a hybrid protein or chimeric protein comprising elements from said type I and II CS proteins. When the latter is fused to the S antigen this will be referred to herein as a hybrid fusion protein.

CSV-S is used herein as a generic term to cover fusion proteins comprising a sequence/fragment form the CS protein of *P. vivax* and a sequence/fragment form the S-antigen of Hepatitis B.

RTS is used herein as a generic term to cover fusion proteins comprising a sequence/fragment form the CS protein of *P.falciparum* and a sequence/fragment form the S-antigen of Hepatitis B.

The hybrid/chimeric protein will generally comprise:
at least one repeat unit derived from the central repeat section of a type I circumsporozoite protein of *P. vivax,* and
at least one repeat unit derived from the central repeating section of a type II circumsporozoite protein of *P. vivax.*

Generally the hybrid protein will also contain an N-terminus fragment from CS protein of *Plasmodium* such as *P. vivax*, for example a fragment comprising region I such as the amino acids shown in SEQ ID No. 1.

Usually the hybrid protein will contain a C-terminus fragment from CS protein of *Plasmodium* such as *P. vivax*, for example a fragment comprising region II such as the motif shown in SEQ ID No 2.

Whilst not wishing to be bound by theory it is thought that the N and C terminal fragments include several T and B cell epitopes.

Any suitable strain of P. *vivax* may be employed in the invention including: Latina, America (ie Sal 1, Belem), Korean, China, Thailand, Indonesia, India, and Vietnam. The construct in SEQ ID No 13 is based on a Korean strain (more specifically a South Korean strain).

P. vivax with type I CS proteins is more prevalent than *P. vivax* with type II CS proteins. Therefore in one aspect the invention employs a CS protein from type I. In an alternative aspect the invention provides a hybrid protein comprising a repeat unit

from type I and a repeat unit from type II, for example wherein more repeat units from type I are included in the hybrid than repeat units of type II.

More specifically the hybrid protein of the invention may include 1 to 15 repeat units such as 9 repeat units from type I.

Examples of suitable repeat units from type I CS proteins are given in SEQ ID No.s 3 to 9.

In one embodiment the invention provides a hybrid with a mixture of different repeat units of type I, such as one of each of those listed in SEQ ID No.s 3 to 9.

One or more repeat units may be duplicated in the hybrid, for example two repeat units of SEQ ID No 3 and/or 4 may be incorporated into the construct.

a) In one aspect the CS protein comprises a unit of SEQ ID No 3.

b) In one aspect the CS protein comprises a unit of SEQ ID No 4, optionally in combination with units as described in paragraph a) directly above.

c) In one aspect the CS protein comprises a unit of SEQ ID No 5, optionally in combination with units as described in paragraph a) or b) directly above.

d) In one aspect the CS protein comprises a unit of SEQ ID No 6, optionally in combination with one or more units as described in paragraphs a) to c) directly above.

f) In one aspect the CS protein comprises a unit of SEQ ID No 7, optionally in combination with one or more units as described in paragraph a) to d) directly above.

g) In one aspect the CS protein comprises a unit of SEQ ID No 8, optionally in combination with one or more units as described in paragraph a) to f) directly above.

h) In one aspect the CS protein comprises a unit of SEQ ID No 9, optionally in combination with one or more units as described in paragraph a) to g) directly above.

Examples of suitable component repeat units from type II CS proteins are given in SEQ ID No.s 10 and 14, such as 10.

In one aspect of the invention there is provided a hybrid protein with 5 or less repeat units derived from type II such as one repeat unit, for example as shown in SEQ ID No. 10.

The hybrid may also include the 12 amino acid insertion found at the end of the repeat region found in certain Asian strains of *P. vivax*, for example as shown in SEQ ID No. 11.

In one embodiment the hybrid protein comprises about 257 amino-acids derived from *P. vivax* CS protein.

The CSV derived antigen component of the invention is generally fused to the amino terminal end of the S protein.

It is believed that the presence of the surface antigen from Hepatitis B boosts the immunogenicity of the CS protein portion, aids stability, and/or assists reproducible manufacturing of the protein.

In one embodiment the hybrid fusion protein comprises about 494 amino acids, for example about 257 of which are derived from *P. vivax* CS protein.

The hybrid fusion protein may also include further antigens derived from *P. falciparium* and/or *P. vivax*, for example wherein the antigen is selected from DBP, PvTRAP, PvMSP2, PvMSP4, PvMSP5, PvMSP6, PvMSP7, PvMSP8, PvMSP9, PvAMA1 and RBP or fragment thereof.

Other example, antigens derived from *P falciparum* include ,PfEMP-1, Pfs 16 antigen, MSP-1, MSP-3, LSA-1, LSA-3, AMA-1 and TRAP. Other *Plasmodium* antigens include *P. falciparum* EBA, GLURP, RAP1, RAP2, Sequestrin, Pf332, STARP, SALSA, PfEXP1, Pfs25, Pfs28, PFS27/25, Pfs48/45, Pfs230 and their analogues in other *Plasmodium* spp.

In an embodiment the hybrid fusion protein (CSV-S) has the amino acid sequence shown in SEQ ID No. 17. In the sequence amino acids 6 to 262 are derived from CSV and 269 to 494 are derived from S. The remaining amino acids are introduced by genetic construction (which, in particular may be varied as appropriate). These four amino acids, Met, Met Ala Pro, are derived specifically from plasmid pGF1-S2 (see Fig. 4)

The properties of the CSV-S fusion protein of SEQ ID No. 17 are provided in the Tables below

Analysis	Whole Protein
Molecular Weight	51794.75 m.w.
Length	494
1 microgram =	19.307 pMoles
Molar Extinction coefficient	90780+/-5%
1 A(280) =	0.57 mg/ml
Isoelectric Point	7.33
Charge at pH 7	1.05

Whole Protein Composition Analysis

Amino Acid(s)	Number countweight	% by	% by frequency
Charged (RKHYCDE)	106	26.35	21.46
Acidic (DE)	38	8.82	7.69
Basic (KR)	39	10.68	7.89
Polar (NCQSTY)	134	28.15	27.13
Hydrophobic (AILFWV)	167	34.68	33.81
A Ala	52	7.14	10.53
C Cys	18	3.58	3.64
D Asp	24	5.33	4.86
E Glu	14	3.49	2.83
F Phe	17	4.83	3.44
G Gly	64	7.05	12.96
H His	4	1.06	0.81
I Ile	17	3.71	3.44
K Lys	20	4.95	4.05
L Leu	42	9.18	8.50
M Met	8	2.03	1.62
N Asn	32	7.05	6.48
P Pro	40	7.50	8.10
Q Gln	21	5.20	4.25
R Arg	19	5.73	3.85
S Ser	30	5.04	6.07
T Thr	26	5.08	5.26
V Val	25	4.78	5.06
W Trp	14	5.03	2.83
Y Tyr	7	2.21	1.42
B Asx	0	0.00	0.00
Z Glx	0	0.00	0.00
X Xxx	0	0.00	0.00
. Ter	1	0.00	0.20

The nucleotide sequence for protein of SEQ ID No 17 is given in SEQ ID No 16.

The component of the protein particles of the invention termed RTS (ie derived from *P.falciparum*) can be prepared as described in WO 93/10152, which includes a description of the RTS* (from *P. falciparum* NF54/3D7 strain).

In one or more embodiments of the invention the antigen derived from *P. falciparum* employed in the fusion protein may be the substantially the whole CS protein thereof.

In one embodiment of the invention full-length S-antigen is employed. In another embodiment a fragment of said S-antigen is employed.

In one embodiment the antigen derived from of *P. falciparum* comprises at least 4 repeat units the central repeat region. More specifically this antigen comprises a sequence which contains at least 160 amino acids, which is substantially homologous to the C-terminal portion of the CS protein. The CS protein may be devoid of the last 12 to 14 (such as 12) amino-acids from the C terminal.

In particular a fusion protein which comprises a portion of the CS protein of *P. falciparum* substantially as corresponding to amino acids 207-395 of the CS protein of *P. falciparum* (strain NF54[3D7]) 7G8 fused in frame via a linear linker to the N-terminal of the S antigen is employed in the particles. The linker may comprise a portion of preS2 from the S-antigen.

More specifically the fusion protein derived from *P. falciparium* employed is that encoded for by the nucleotide sequence for the RTS expression cassette, provide in SEQ ID No 18.

Suitable S antigens, may comprise a preS2. An example of a suitable serotype is adw (Nature 280:815-819, 1979).

In one aspect the hybrid fusion proteins of the invention comprise a portion derived from a mutant s protein, for example as described in published US application No. 2006/194196 (also published as WO 2004/113369). This document describes a mutant labeled HDB05. In particular it describes comparisons of the mutant and wild type proteins in Figures 1 and 6 and genes for the mutant in figures 4 and 5. Sequence 12 to 22 therein describe particular polypeptides of the mutant S protein. Each of the above is incorporated herein by reference.

The fusion protein CSV-S may for example be prepared employing the plasmid pGF1-S2 (see Fig. 2 and the examples for further details), which when the appropriate sequence corresponding to CSV is inserted at the SamI cloning site can under suitable conditions produce the fusion protein CSV-S.

The DNA sequences encoding the proteins of the present invention are, in one embodiment flanked by transcriptional control elements, preferably derived from yeast genes and incorporated into an expression vector.

An expression cassette for hybrid proteins of the invention may, for example, be constructed comprising the following features:

- A promoter sequence, derived, for example, from the *S.cerevisiae TDH3* gene.
- A sequences encoding for an appropriate fusion protein.
- A transcription termination sequence contained within the sequence, derived, for example, from the *S. cerevisiae ARG3* gene.

An example of a specific promoter is the promoter from the *S. cerevisiae TDH3* gene Musti *et al.*

The invention also extends to vectors employed in the preparation of the hybrid fusion protein.

A suitable plasmid can then be employed to insert the sequence encoding for the hybrid fusion protein into a suitable host for synthesis. An example of a suitable plasmid is pRIT15546 a 2 micron-based vector for carrying a suitable expression cassette, see Fig 1 and Examples for further details.

The plasmid will generally contain an in-built marker to assist selection, for example a gene encoding for antibiotic resistance or LEU2 or HIS auxotrophy.

Generally the host will have an expression cassette for each fusion protein in the particle and may also have one or more expression cassettes for the S antigen integrated in its genome.

The invention also relates to a host cell transformed with a vector according to the invention. Host cells can be prokaryotic or eukaryotic but preferably, are yeast, for example *Saccharomyces* (for example *Saccharomyces cerevisiae* such as DC5 in ATCC data base (accession number 20820), under the name RIT DC5 cir(o). Depositor: Smith Kline-RIT) and *non- Saccharomyces* yeasts. These include *Schizosaccharomyces* (eg *Schizosaccharomyces pombe) Kluyveromyces (eg Kluyveromyces lactis*), *Pichia* (eg *Pichia pastoris), Hansenula* (eg *Hansenula polymorpha), Yarrowia (*eg *Yarrowia lipolytica*) and *Schwanniomyces* (eg *Schwanniomyces occidentalis*).

In one aspect the invention relates to a recombinant yeast strain Y1834 (and use thereof), for expressing the fusion protein, see Examples for preparation of the same.

In another embodiment the invention provides a recombinant yeast strain Y1835 or Y1845 (and use of same) for expressing fusion protein of the invention, see Examples for further details.

The nucleotide sequences or part thereof (such as the portion encoding the CS/hybrid protein but optionally not the portion encoding protein S) employed herein may be codon-optimized for expression in a host, such as yeast.

The invention also extends to a host comprising a polynucleotide such as DNA encoding for two or more components of the particle, employed in the present invention.

In one embodiment the host cell comprises an expression cassette for a fusion protein derived from *P. vivax* and an expression cassette for the fusion protein derived from *P. falciparum.*

In certain hosts, such as yeast cells, once expressed the fusion protein (comprising the S antigen) is spontaneously assembled into a protein structure/particle composed of numerous monomers of said fusion proteins. When the yeast expresses two different fusion proteins these are believed to be co-assembled in particles.

When the chosen recipient yeast strain already carries in its genome several integrated copies of Hepatitis B S expression cassettes then the particles assembled may also include monomers of unfused S antigen.

These particles may also be referred to a Virus Like Particles (VLP). The particles may also be described as multimeric lipoprotein particles.

Alternatively these particles can be prepared in a number of ways, for example by fusing each the *Plasmodium* derived antigens to another fusion partner, (for example the antigens of Hepatitis B virus or a viral structural protein) and expressing the same in a suitable host such as yeast or bacteria.

Thus there is provided an immunogenic protein particle comprising the following monomers:

a. a fusion protein comprising sequences derived from a CS protein of *P.vivax,* and
b. a fusion protein comprising sequences derived from CS protein of *P. falciparum.*

In a further aspect the invention provides a fusion protein comprising:

a) a sequence derived from a CS protein of P vivax (such as a sequence from the repeat region of type I and/or type II)
b) a sequence derived from the CS protein of P. falciparum (such as a sequence from the repeat region thereof), and
c) a sequence from the S-antigen of Hepatitis B.

Thus the invention extends to a protein particle comprising a fusion protein derived from CS protein of *P.vivax* and a fusion protein derived from CS protein of P. *falciparium*, wherein the antigen fused with the *Plasomdium* antigen is chosen to induce the formation/assembly of the lipoparticles when said fusion proteins are expressed in a suitable host.

Thus the invention provides a VLP comprising CSV-S and RTS units. In one aspect the invention provides a particle consisting essentially of CSV-S and RTS units. In an alternative aspect the particles produced comprise or consist of essentially of CSV-S, RTS and S units.

Various approaches can be used to engineer yeast for the preparation of said particles, for example an expression cassette for the fusion protein can be inserted into the genome of a yeast already containing an expression cassette for one of the required

fusion proteins and/or the S antigen. However, a skilled person working in the field is well able to prepare a suitable host for the preparation of particles according to the invention.

Thus in one aspect the invention provides a suitable host such as yeast comprising DNA encoding for CSV-S, RTS and optionally S. In an alternative aspect a suitable host is, one or more plasmids capable of expressing CSV-S, RTS and optionally S. The plasmid may, for example be used to express the protein in conjunction with, for example, a yeast.

Whilst not wishing to be bound by theory it is thought that the surfactants used to liberate the protein from the cells may also assist in the stabilization of the lipoprotein particles.

It is hypothesized that the lipoprotein particles of the invention may contribute to further stimulating *in vivo* the immune response to the antigenic protein(s).

In one aspect the invention provides a replication deficient viral vector encoding a one or more CS proteins, for example which correspond(s) to one or more CS proteins comprised in the particles according the invention.

Suitable viral vectors may be derived from adeno viral vectors, adeno-associated viral vectors (AAVs), measles, lentiviruses, alphaviruses, bacloviruses, herpes simplex virus, and poxviruses such as cowpox, fowlpox, pigeonpox, canarypox, suipox and sheeppox/goatpox. Methodology for preparing adeno viral vectors encoding a malaria antigen is, for example, described in WO 2004/055187.

The protein encoded by the vector may, for example, be modified to prevent glycosylation of the protein during expression, for example certain serines may be replaced by alanine residues to reduce glycosylation.

Viral vectors employed in the invention may be recombinant.

Adenovirus

Adenoviral vectors of the present invention comprise one or more heterologous polynucleotides (DNA) which encode one or more immunogenic polypeptides.

Adenoviral vectors of use in the present invention may be derived from a range of mammalian hosts.

Adenoviruses (herein referred to as "Ad" or "Adv") have a characteristic morphology with an icosohedral capsid consisting of three major proteins, hexon (II), penton base (III) and a knobbed fibre (IV), along with a number of other minor proteins, VI, VIII, IX, IIIa and IVa2 (Russell W.C. 2000, Gen Viriol, 81:2573-2604). The virus genome is a linear, double-stranded DNA with a terminal protein attached covalently to the 5' termini, which have inverted terminal repeats (ITRs). The virus DNA is intimately

associated with the highly basic protein VII and a small peptide termed mu. Another protein, V, is packaged with this DNA-protein complex and provides a structural link to the capsid via protein VI. The virus also contains a virus-encoded protease, which is necessary for processing of some of the structural proteins to produce mature infectious virus.

Over 100 distinct serotypes of adenovirus have been isolated which infect various mammalian species, 51 of which are of human origin. Thus one or more of the adenoviral vectors may be derived from a human adenovirus. Examples of such human-derived adenoviruses are Ad1, Ad2, Ad4, Ad5, Ad6, Ad11, Ad 24, Ad34, Ad35, Ad50/51 particularly Ad5, Ad11 and Ad35. The human serotypes have been categorised into six subgenera (A-F) based on a number of biological, chemical, immunological and structural criteria.

Although Ad5-based vectors have been used extensively in a number of gene therapy trials, there may be limitations on the use of Ad5 and other group C adenoviral vectors due to preexisting immunity in the general population due to natural infection. Ad5 and other group C members tend to be among the most seroprevalent serotypes. Immunity to existing vectors may develop as a result of exposure to the vector during treatment. These types of preexisting or developed immunity to seroprevalent vectors may limit the effectiveness of gene therapy or vaccination efforts. Alternative adenovirus serotypes, thus constitute very important targets in the pursuit of gene delivery systems capable of evading the host immune response.

One such area of alternative serotypes are those derived from non human primates, especially chimpanzee adenoviruses. See US Patent 6,083,716 which describes the genome of two chimpanzee adenoviruses.

It has been shown that chimpanzee ("Pan" or "C") adenoviral vectors induce strong immune responses to transgene products as efficiently as human adenoviral vectors (Fitzgerald et al. J. Immunol. 170:1416).

Non-human primate adenoviruses can be isolated from the mesenteric lymph nodes of chimpanzees. Chimpanzee adenoviruses are sufficiently similar to human adenovirus subtype C to allow replication of E1 deleted virus in HEK 293 cells. Yet chimpanzee adenoviruses are phylogenetically distinct from the more common human serotypes (Ad2 and Ad5). Pan 6 is less closely related to and is serologically distinct from Pans 5, 7 and 9.

Thus one or more of the adenoviral vectors may be derived from a non-human primate adenovirus eg a chimpanzee adenovirus such as one selected from serotypes Pan5, Pan6, Pan7 and Pan9.

Adenoviral vectors may also be derived from more than one adenovirus serotype, and each serotype may be from the same or different source. For example they may be derived from more than one human serotype and/or more than one non-human

primate serotype. Methods for constructing chimeric adenoviral vectors are disclosed in WO2005/001103.

There are certain size restrictions associated with inserting heterologous DNA into adenoviruses. Human adenoviruses have the ability to package up to 105% of the wild type genome length (Bett et al 1993, J Virol 67 (10), 5911-21). The lower packaging limit for human adenoviruses has been shown to be 75% of the wild type genome length (Parks et al 1995, J Virol 71(4), 3293-8).

One example of adenoviruses useful in the present invention are adenoviruses which are distinct from prevalent naturally occurring serotypes in the human population such as Ad2 and Ad5. This avoids the induction of potent immune responses against the vector which limits the efficacy of subsequent administrations of the same serotype by blocking vector uptake through neutralizing antibody and influencing toxicity.

Thus, the adenovirus may be an adenovirus which is not a prevalent naturally occurring human virus serotype. Adenoviruses isolated from animals have immunologically distinct capsid, hexon, penton and fibre components but are phylogenetically closely related. Specifically, the virus may be a non-human adenovirus, such as a simian adenovirus and in particular a chimpanzee adenovirus such as Pan 5, 6, 7 or 9. Examples of such strains are described in WO 03/000283 and are available from the American Type Culture Collection, 10801 University Boulevard, Manassas, Virginia 20110-2209, and other sources. Desirable chimpanzee adenovirus strains are Pan 5 [ATCC VR-591], Pan 6 [ATCC VR-592], and Pan 7 [ATCC VR-593].

Use of chimpanzee adenoviruses is thought to be advantageous over use of human adenovirus serotypes because of the lack of pre-existing immunity, in particular the lack of cross-neutralising antibodies, to adenoviruses in the target population. Cross-reaction of the chimpanzee adenoviruses with pre-existing neutralizing antibody responses is only present in 2% of the target population compared with 35% in the case of certain candidate human adenovirus vectors. The chimpanzee adenoviruses are distinct from the more common human subtypes Ad2 and Ad5, but are more closely related to human Ad4 of subgroup E, which is not a prevalent subtype. Pan 6 is less closely related to Pan 5, 7 and 9.

The adenovirus of the invention may be replication defective. This means that it has a reduced ability to replicate in non-complementing cells, compared to the wild type virus. This may be brought about by mutating the virus e.g. by deleting a gene involved in replication, for example deletion of the E1a, E1b, E3 or E4 gene.

The adenoviral vectors in accordance with the present invention may be derived from replication defective adenovirus comprising a functional E1 deletion. Thus the adenoviral vectors according to the invention may be replication defective due to the absence of the ability to express adenoviral E1a and E1b, i.e., are functionally deleted in E1a and E1b. The recombinant adenoviruses may also bear functional deletions in

other genes [see WO 03/000283] for example, deletions in E3 or E4 genes (or part thereof such as part of E3). The adenovirus delayed early gene E3 may be eliminated from the adenovirus sequence which forms part of the recombinant virus. The function of E3 is not necessary to the production of the recombinant adenovirus particle. Thus, it is unnecessary to replace the function of this gene product in order to package a recombinant adenovirus useful in the invention. In one particular embodiment the recombinant adenoviruses have functionally deleted E1 and E3 genes. The construction of such vectors is described in Roy et al., Human Gene Therapy 15:519-530, 2004. In one aspect the adeno virus has E1 and E4 deleted and part of E3 deleted.

Recombinant adenoviruses may also be constructed having a functional deletion of the E4 gene, although it may be desirable to retain the E4 ORF6 function. Adenovirus vectors according to the invention may also contain a deletion in the delayed early gene E2a. Deletions may also be made in any of the late genes L1 through to L5 of the adenovirus genome. Similarly deletions in the intermediate genes IX and IVa may be useful.

Other deletions may be made in the other structural or non-structural adenovirus genes. The above deletions may be used individually, i.e. an adenovirus sequence for use in the present invention may contain deletions of E1 only. Alternatively, deletions of entire genes or portions thereof effective to destroy their biological activity may be used in any combination. For example in one exemplary vector, the adenovirus sequences may have deletions of the E1 genes and the E4 gene, or of the E1, E2a and E3 genes, or of the E1 and E3 genes (such as functional deletions in E1a and E1b, and a deletion of at least part of E3), or of the E1, E2a and E4 genes, with or without deletion of E3 and so on. Such deletions may be partial or full deletions of these genes and may be used in combination with other mutations, such as temperature sensitive mutations to achieve a desired result.

The adenoviral vectors can be produced on any suitable cell line in which the virus is capable of replication. In particular, complementing cell lines which provide the factors missing from the viral vector that result in its impaired replication characteristics (such as E1 and/or E4) can be used. Without limitation, such a cell line may be HeLa [ATCC Accession No. CCL 2], A549 [ATCC Accession No. CCL 185], HEK 293, KB [CCL 17], Detroit [*e.g.*, Detroit 510, CCL 72] and WI-38 [CCL 75] cells, among others. These cell lines are all available from the American Type Culture Collection, 10801 University Boulevard, Manassas, Virginia 20110-2209. Other suitable parent cell lines may be obtained from other sources, such as PER.C6© cells, as represented by the cells deposited under ECACC no. 96022940 at the European Collection of Animal Cell Cultures (ECACC) at the Centre for Applied Microbiology and Research (CAMR, UK) or Her 96 cells (Crucell).

The invention extends to use of known cell lines for the preparation of a viral vector encoding a protein of the present invention.

The polynucleotide sequences which encode immunogenic CS polypeptides may be codon optimised for mammalian cells. Such codon-optimisation is described in detail in WO 05/025614.

The present invention also relates to vaccines comprising an immunoprotective amount of protein particle according to the invention in admixture with a suitable diluent or carrier.

The invention also extend to a composition comprising a particle according to the invention and a viral vector comprising a malaria antigen, particularly a malaria antigen common with said particle, and optionally an adjuvant.

In the context of this specification excipient, refers to a component in a pharmaceutical formulation with no therapeutic effect in its own right. A diluent or carrier falls within the definition of an excipient.

Immunogenic in the context of this specification is intended to refer to the ability to illicit an immune response. This response may, for example be when the lipoprotein particle is administered in an appropriate formulation which may include/require a suitable adjuvant. A booster comprising a dose similar or less than the original dose may be required to obtain the required immunogenic response.
The composition/pharmaceutical formulations according to the invention may also include in admixture one or more further antigens such as those derived from *P. falciparium* and/or *P. vivax*, for example wherein the antigen is selected from DBP, PvTRAP, PvMSP2, PvMSP4, PvMSP5, PvMSP6, PvMSP7, PvMSP8, PvMSP9, PvAMA1 and RBP or fragment thereof.

Other example, antigens derived from *P falciparum* include ,PfEMP-1, Pfs 16 antigen, MSP-1, MSP-3, LSA-1, LSA-3, AMA-1 and TRAP. Other *Plasmodium* antigens include *P. falciparum* EBA, GLURP, RAP1, RAP2, Sequestrin, Pf332, STARP, SALSA, PfEXP1, Pfs25, Pfs28, PFS27/25, Pfs48/45, Pfs230 and their analogues in other *Plasmodium* spp.

The compositions/pharmaceutical formulations according to the invention may also comprise particles of RTS, S (as described in WO 93/10152) in admixture with the particles according to the invention.

In one embodiment the viral vector construct is as described in WO 2004/055187.

In the vaccine of the invention, an aqueous solution of the particle may be used directly. Alternatively, the protein with or without prior lyophilisation can be mixed or absorbed with any of the known adjuvants which include but are not limited to alum, muramyl dipeptide, saponins such as Quil A.

Particular adjuvants are those selected from the group of metal salts, oil in water emulsions, Toll like receptors agonist, (in particular Toll like receptor 2 agonist, Toll

like receptor 3 agonist, Toll like receptor 4 agonist, Toll like receptor 7 agonist, Toll like receptor 8 agonist and Toll like receptor 9 agonist), saponins or combinations thereof with the proviso that metal salts are only used in combination with another adjuvant and not alone unless they are formulated in such a way that not more than about 60% of the antigen is adsorbed onto the metal salt. More specifically, not more than about 50%, for example 40% of the antigen is adsorbed onto the metal salt, and in one embodiment not more than about 30% of the antigen is adsorbed onto the metal salt. The level of antibody adsorbed onto the metal salt may be determined by techniques well known in the art. The level of free antigen may be increased by, for example, formulating the composition in the presence of phosphate ions, such as phosphate buffered saline, or by increasing the ratio of antigen to metal salt. In one embodiment the adjuvant does not include a metal salt as sole adjuvant. In one embodiment the adjuvant does not include a metal salt.

In an embodiment the adjuvant is a Toll like receptor (TLR) 4 ligand, preferably an agonist such as a lipid A derivative particularly monophosphoryl lipid A or more particularly 3 Deacylated monophoshoryl lipid A (3D – MPL).

3 Deacylated monophosphoryl lipid A is known from US patent No. 4,912,094 and UK patent application No. 2,220,211 (Ribi) and is available from Ribi Immunochem, Montana, USA.

3D –MPL is sold under the trademark MPL® by Corixa corporation and primarily promotes CD4+ T cell responses with an IFN-g (Th1) phenotype. It can be produced according to the methods disclosed in GB 2 220 211 A. Chemically it is a mixture of 3-deacylated monophosphoryl lipid A with 3, 4, 5 or 6 acylated chains. Preferably in the compositions of the present invention small particle 3D- MPL is used. Small particle 3D -MPL has a particle size such that it may be sterile-filtered through a 0.22μm filter. Such preparations are described in International Patent Application No. WO 94/21292. Synthetic derivatives of lipid A are known and thought to be TLR 4 agonists including, but not limited to:

OM174 (2-deoxy-6-O-[2-deoxy-2-[(R)-3-dodecanoyloxytetra-decanoylamino]-4-o-phosphono-β-D-glucopyranosyl]-2-[(R)-3-hydroxytetradecanoylamino]-α-D-glucopyranosyldihydrogenphosphate), (WO 95/14026)

OM 294 DP (3S, 9 R) –3-[(R)-dodecanoyloxytetradecanoylamino]-4-oxo-5-aza-9(R)-[(R)-3-hydroxytetradecanoylamino]decan-1,10-diol,1,10-bis(dihydrogenophosphate) (WO99 /64301 and WO 00/0462)

OM 197 MP-Ac DP (3S-, 9R) -3-[(R) -dodecanoyloxytetradecanoylamino]-4-oxo-5-aza-9-[(R)-3-hydroxytetradecanoylamino]decan-1,10-diol,1 -dihydrogenophosphate 10-(6-aminohexanoate) (WO 01/46127).

Typically when 3D-MPL is used the antigen and 3D-MPL are delivered with alum or presented in an oil in water emulsion or multiple oil in water emulsions. The

incorporation of 3D-MPL is advantageous since it is a stimulator of effector T-cells responses.

Other TLR4 ligands which may be used are alkyl Glucosaminide phosphates (AGPs) such as those disclosed in WO 9850399 or US 6303347 (processes for preparation of AGPs are also disclosed), or pharmaceutically acceptable salts of AGPs as disclosed in US 6764840. Some AGPs are TLR4 agonists, and some are TLR4 antagonists. Both are thought to be useful as adjuvants.

Another immunostimulant for use in the present invention is Quil A and its derivatives. Quil A is a saponin preparation isolated from the South American tree *Quilaja Saponaria Molina* and was first described as having adjuvant activity by Dalsgaard *et al.* in 1974 ("Saponin adjuvants", Archiv. für die gesamte Virusforschung, Vol. 44, Springer Verlag, Berlin, p243-254). Purified fragments of Quil A have been isolated by HPLC which retain adjuvant activity without the toxicity associated with Quil A (EP 0 362 278), for example QS7 and QS21 (also known as QA7 and QA21). QS-21 is a natural saponin derived from the bark of *Quillaja saponaria* Molina which induces CD8+ cytotoxic T cells (CTLs), Th1 cells and a predominant IgG2a antibody response.

Particular formulations of QS21 have been described which further comprise a sterol (WO 96/33739). The ratio of QS21: sterol will typically be in the order of 1:100 to 1 : 1 weight to weight. Generally an excess of sterol is present, the ratio of QS21 : sterol being at least 1 : 2 w/w. Typically for human administration QS21 and sterol will be present in a vaccine in the range of about 1 μg to about 100 μg, such as about 10 μg to about 50 μg per dose.

The liposomes generally contain a neutral lipid, for example phosphatidylcholine, which is usually non-crystalline at room temperature, for example eggyolk phosphatidylcholine, dioleoyl phosphatidylcholine or dilauryl phosphatidylcholine. The liposomes may also contain a charged lipid which increases the stability of the lipsome-QS21 structure for liposomes composed of saturated lipids. In these cases the amount of charged lipid is often 1-20% w/w, such as 5-10%. The ratio of sterol to phospholipid is 1-50% (mol/mol), such as 20-25%.

These compositions may contain MPL (3-deacylated mono-phosphoryl lipid A, also known as 3D-MPL). 3D-MPL is known from GB 2 220 211 (Ribi) as a mixture of 3 types of De-O-acylated monophosphoryl lipid A with 4, 5 or 6 acylated chains and is manufactured by Ribi Immunochem, Montana. .

The saponins may be separate in the form of micelles, mixed micelles (generally, but not exclusively with bile salts) or may be in the form of ISCOM matrices (EP 0 109 942), liposomes or related colloidal structures such as worm-like or ring-like multimeric complexes or lipidic/layered structures and lamellae when formulated with cholesterol and lipid, or in the form of an oil in water emulsion (for example as in WO

95/17210). The saponins may often be associated with a metallic salt, such as aluminium hydroxide or aluminium phosphate (WO 98/15287).

Usually, the saponin is presented in the form of a liposome, ISCOM or an oil in water emulsion.

Immunostimulatory oligonucleotides may also be used. Examples oligonucleotides for use in adjuvants or vaccines of the present invention include CpG containing oligonucleotides, generally containing two or more dinucleotide CpG motifs separated by at least three, more preferably at least six or more nucleotides. A CpG motif is a Cytosine nucleotide followed by a Guanine nucleotide. The CpG oligonucleotides are typically deoxynucleotides. In one embodiment the internucleotide in the oligonucleotide is phosphorodithioate, or more preferably a phosphorothioate bond, although phosphodiester and other internucleotide bonds are within the scope of the invention. Also included within the scope of the invention are oligonucleotides with mixed internucleotide linkages. Methods for producing phosphorothioate oligonucleotides or phosphorodithioate are described in US 5,666,153, US 5,278,302 and WO 95/26204.

Examples of oligonucleotides are as follows:
TCC ATG ACG TTC CTG ACG TT (CpG 1826)
TCT CCC AGC GTG CGC CAT (CpG 1758)
ACC GAT GAC GTC GCC GGT GAC GGC ACC ACG
TCG TCG TTT TGT CGT TTT GTC GTT (CpG 2006)
TCC ATG ACG TTC CTG ATG CT (CpG 1668)
TCG ACG TTT TCG GCG CGC GCC G (CpG 5456),
the sequences may contain phosphorothioate modified internucleotide linkages.

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

The CpG oligonucleotides may be synthesized by any method known in the art (for example see EP 468520). Conveniently, such oligonucleotides may be synthesized utilising an automated synthesizer.

Examples of a TLR 2 agonist include peptidoglycan or lipoprotein. Imidazoquinolines, such as Imiquimod and Resiquimod are known TLR7 agonists. Single stranded RNA is also a known TLR agonist (TLR8 in humans and TLR7 in mice), whereas double stranded RNA and poly IC (polyinosinic-polycytidylic acid - a commercial synthetic mimetic of viral RNA) are exemplary of TLR 3 agonists. 3D-MPL is an example of a TLR4 agonist whilst CpG is an example of a TLR9 agonist.

An immunostimulant may alternatively or in addition be included. In a one embodiment this immunostimulant will be 3 Deacylated monophosphoryl lipid A (3D-MPL).

In one aspect the adjuvant comprises 3D-MPL.

In one aspect the adjuvant comprises QS21.

In one aspect the adjuvant comprises CpG.

In one aspect the adjuvant is formulated as an oil in water emulsion.

In one aspect the adjuvant is formulated as liposomes.

Adjuvants combinations include 3D-MPL and QS21 (EP 0 671 948 B1), oil in water emulsions comprising 3D-MPL and QS21 (WO 95/17210, WO 98/56414), or 3D-MPL formulated with other carriers (EP 0 689 454 B1). Other preferred adjuvant systems comprise a combination of 3D-MPL, QS21 and a CpG oligonucleotide as described in US 6558670 and US 6544518.

In one embodiment of the present invention provides a vaccine comprising a particleas herein described, in combination with 3D-MPL and a carrier. Typically the carrier will be an oil in water emulsion or alum.

The protein particles of the present invention may also be encapsulated into microparticles such as liposomes.

Vaccine preparation is generally described in New Trends and Developments in Vaccines, edited by Voller et al., University Park Press, Baltimore, Maryland, U.S.A., 1978. Encapsulation within liposomes is described, for example, by Fullerton, U.S. Patent 4,235,877.

The amount of the protein particles of the present invention present in each vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccines. Such amount will vary depending upon which specific immunogen is employed and whether or not the vaccine is adjuvanted. Generally, it is expected that each does will comprise 1-1000μg of protein, preferably 1-200 μg most preferably 10-100μg. An optimal amount for a particular vaccine can be ascertained by standard studies involving observation of antibody titres and other responses in subjects. Following an initial vaccination, subjects will preferably receive a boost in about 4 weeks, followed by repeated boosts every six months for as long as a risk of infection exists. The immune response to the protein of this invention is enhanced by the use of adjuvant and or an immuno stimulant.

The amount of 3D MPL used is generally small, but depending on the vaccine formulation may be in the region of 1-1000μg per dose, for example 1-500μg per dose, and such as between 1 to 100μg per dose.

The amount of CpG or immunostimulatory oligonucleotides in the adjuvants or vaccines of the present invention is generally small, but depending on the vaccine formulation may be in the region of 1-1000μg per dose, preferably 1-500μg per dose, and more preferably between 1 to 100μg per dose.

The amount of saponin for use in the adjuvants of the present invention may be in the region of 1-1000μg per dose, preferably 1-500μg per dose, more preferably 1-250μg per dose, and most preferably between 1 to 100μg per dose.

The formulations of the present invention may be used for both prophylactic and therapeutic purposes. Therapy includes prophylactic treatment. Accordingly the invention provides a vaccine composition as described herein for use in medicine, for example, for the treatment (including phrophylaxis) of malaria.

A further aspect of the present invention is to provide a process for the preparation of hybrid protein of the invention, which process comprises expressing DNA sequence encoding the protein, in a suitable host, preferably a yeast, and recovering the product.

A further aspect of the invention lies in a method of treating a patient susceptible to plasmodium infections by administering an effective amount of a vaccine as hereinbefore described.
In a further aspect there is provided a combination for treatment comprising:
an immunogenic particle according to the invention an a viral vector encoding for a malaria antigen, such as said hybrid antigen.

For example wherein said particle and said viral vector are administered concomitantly, for example they may be admixed for administration simultaneously or alternatively may formulated for administration sequentially.

As used herein the term "concomitantly" means wherein said combination of components are administered within a period of no more than 12 hours eg within a period of no more than 1 hour, typically on one occasion e.g. in the course of the same visit to the health professional, for example where they are administered sequentially or simultaneously.

The invention also includes prime boost regimes with the various components described herein, for example priming with viral vector and boosting with said particles or *vice versa*.

In the context of this specification comprising is to be interpreted as including.

Aspects of the invention comprising a certain element are also intended to extend to said aspects consisting or consisting essentially of the relevant elements.

The examples below are shown to illustrate the methodology, which may be employed to prepare particles of the invention. The examples may or may not form an aspect of the invention.

EXAMPLES

Example 1

DESCRIPTION OF STRAIN Y1834

The yeast recombinant strain Y1834 may be used to express the fusion protein. It consists of the *Saccharomyces cerevisiae* host strain DC5 transformed with the recombinant expression vector pRIT15546.

DC5 is a laboratory yeast strain (ATCC No: 20820) with the following genotype: leu2-3, leu2-112, his3, can1-11. The double leu-2 mutation permits selection for the uptake and maintenance of the pRIT15546 vector which carries a functional LEU-2 gene copy. Only those cells carrying a vector with a LEU-2 gene can grow when leucine is absent from the growth medium.

The vector pRIT15546 is a yeast episomal expression vector (2μ-based vector) carrying the expression cassette. The recombinant expression is driven by a promoter derived from the yeast TDH3 gene (constitutive expression). The construction of pRIT15546 vector is detailed below.

Construction of pRIT15546 vector.

A synthetic gene, with an appropriate codon usage for yeast expression is constructed and sub-cloned into pUC57 vector. The resulting plasmid pUC57/CSV and the yeast expression vector pGf1-S2 are both restricted with the appropriate enzyme. The vector pGf1-S2 was constructed (at GSK) by a multistep cloning procedure. This vector, which already carries an S expression cassette, allows the construction of fusion genes, as N-terminal in-frame fusion with the S gene of Hepatitis B virus. The final expression vector, after sequence verification, was named pRIT15546 (Fig 3)

Transformation of strain DC5.

The leu- and his-auxotrophic DC5 strain is transformed with the recombinant plasmid pRIT15546, by using yeast standard protocol. Transformed cells were plated on agar selective plates. One transformant was selected and received the official designation Y1834.

EXPRESSION OF THE RECOMBINANT PROTEIN:

Y1834 is grown, at 30°C, in **YNB** (Yeast Nitrogen Base available from Kracker Scientific Inc) minimal medium supplemented with 8μg/ml histidine to an O.D.(620nm) of 0.5. Then cells are harvested and cellular extracts are prepared.

EXTRACT PREPARATION:

Cells are resuspended in Breaking Buffer and mechanically disrupted (glass beads). Extract is centrifuged for 15 minutes at 5000 rpm. Supernatant fraction is run on SDS – PAGE 4-20%.

Breaking Buffer:	50mM phosphate Na buffer(PH:7.5) 4mMEDTA Tween-20 0.5% + proteases inhibitor cocktail (Complete/ ROCHE)
Cell concentration:	100 ml culture (OD:0.5) in 5 ml breaking buffer = concentration of 10 OD unit /ml.
Crude extract clarification:	extract centrifuged 15 minutes/5000 rpm

DETECTION OF RECOMBINANT PROTEIN

Clarified extracts are run on SDS-PAGE 4-20%, proteins transferred to nitrocellulose membrane and subjected to immunostaining

Western blot analysis:

Reagent = Mouse monoclonal antibody anti-S (prepared by GSK Biologicals)- (dilution: 1/500)

Anti-S antibodies which are commercially available may be substituted for those employed in this method. Alternatively anti-CSV antibodies may be employed, for example those known as MR4 available from NIH.

Example 2:

DESCRIPTION OF STRAIN Y1835

The yeast recombinant strain Y1835 simultaneously expresses the CSV-S fusion protein and the S antigen. To obtain a strain co-expressing CSV-S and S proteins, the *Saccharomyces cerevisiae* strain Y1295, which already carries five integrated copies of S expression cassettes, was transformed with the recombinant integrative expression vector pRIT15582.

The strain Y1295 was constructed at GSK by a multistep transformation procedure. The construction of Y1295 strain is described in WO 93/10152. Strain Y1295 has the following genotype: leu2-3, leu2-112, gal1. The leu-2 mutation permits selection for the uptake of pRIT15582-derived linear DNA fragment which carries the CSV-S cassette and the functional LEU2 gene.

The vector pRIT15582 is a yeast integrative expression vector (Ty-based vector) carrying the CSV-S expression cassette. The recombinant expression is driven by a promoter derived from the yeast TDH3 gene (constitutive expression). The construction of pRIT15582 vector is detailed below.

Construction of pRIT15582 integrative vector.

The starting material used to construct pRIT15582 vector was the expression plasmid pRIT15546 (Fig1). The construction of this plasmid is described in example 1. Digestion of pRIT 15546 with *HindIII* endonuclease liberates a 3706 bp long DNA fragment corresponding to the complete CSV-S expression cassette (pTDH3 + CSV-S + tARG3).This *HindIII* DNA fragment (after filling with T4 DNA polymerase) was inserted on the Ty-based integrative vector pRIT13144 at the unique *SalI* cloning site (SalI restricted/T4 treated).The resulting plasmid pRIT15582 contains, in addition to the expression cassette, the yeast LEU2 gene as selective marker (Fig 3). Digestion of pRIT15582 with *XhoI* endonuclease liberates a 8500 bp linear fragment shown in figure 4 which can be integrated into the yeast genome by homologous recombination of the free ends with resident Ty elements.

Transformation of strain Y1295.

To obtain a strain expressing both S and CSV-S proteins, strain Y1295 was transformed with the 8500bp linear *XhoI* fragment (Fig 4) with selection for Leu+ colonies. Several integrants containing sets of both expression cassettes present in the genome at various ratio were obtained. One transformant carrying four copies of CSV-S cassettes was selected and given the official designation Y1835.

EXPRESSION OF THE RECOMBINANT PROTEIN:

Y1835 is grown, at 30°C, in **YNB** (Yeast Nitrogen Base available from Kracker Scientific Inc) minimal medium to an O.D (620nm) of about 0.5 (0.8). Then cells are harvested and cellular extracts are prepared.

ANALYSIS OF EXPRESSION PRODUCTS BY IMMUNOBLOTTING:

EXTRACT PREPARATION:

Cells are re-suspended in Breaking Buffer and mechanically disrupted (glass beads). Extract is centrifuged for 5-10 minutes at 5000 rpm. Supernatant fraction is run on SDS – PAGE 12.5%.

Breaking Buffer:	50mM phosphate Na buffer (PH:7.5) 4mMEDTA Tween-20 0.5% + proteases inhibitor cocktail (Complete/ ROCHE)

Cell concentration: 100 ml culture (OD:0.5) in 2.5 ml breaking buffer = concentration of 20 OD unit/ml.

Crude extract clarification: extract centrifuged 5-10 minutes/5000 rpm

DETECTION OF RECOMBINANT PROTEIN

Clarified extracts are run on SDS-PAGE 12.5%, proteins transferred to nitrocellulose membrane and subjected to immunostaining

Western blot analysis (Fig 5):

Reagents : 1/Mouse monoclonal antibody anti-S (prepared by GSK Biologicals)- (dilution: 1/250)
2/ Rabbit polyclonal antibody anti-CSV (kindly provided by WRAIR)-dilution 1/20,000.

Anti-S antibodies as well as anti-*P.vivax*/CSP antibodies which are commercially available may be substituted for those employed in this method.

Example 3:

DESCRIPTION OF STRAIN Y1845

The yeast recombinant strain Y1845 simultaneously expresses the CSV-S fusion protein, the RTS fusion and the S antigen. To obtain a strain co-expressing CSV-S, RTS and S proteins, the *Saccharomyces cerevisiae* strain Y1295, which already carries five integrated copies of S expression cassettes, was transformed with an equimolar solution of RTS and CSV-S linear DNA fragments derived from pRIT13540 and pRIT15582 integrative vector, respectively.

The strain Y1295 was constructed at GSK by a multistep transformation procedure. The construction of Y1295 strain is described in WO 093/ 10152 file. Strain Y1295 has the following genotype: leu2-3, leu2-112, gal1. The leu-2 mutation permits selection for the uptake of pRIT15582-derived linear DNA fragment which carries the CSV-S cassette and the functional LEU2 gene.

The vectors pRIT13540 and pRIT15582 are yeast integrative expression vectors (Ty-based vector) carrying the RTS and CSV-S expression cassettes, respectively. For both vectors, the recombinant expression is driven by a promoter derived from the yeast TDH3 gene (constitutive expression). The construction of pRIT15582 vector is detailed in example 2. The construction of pRIT13540 vector is described in WO 93/ 10152 file.

Preparation of the linear integrative DNA fragments.

The integrative vectors pRIT13540 and pRIT15582 were both restricted with *XhoI* endonuclease, liberating a 8200 bp and a 8500bp DNA fragments, respectively. These fragments can be integrated into the yeast genome by homologous recombination of the free ends with resident Ty elements.

Transformation of strain Y1295.

To obtain a strain expressing the three RTS, CSV-S and S proteins, strain Y1295 was transformed with an equimolar solution of the 8200bp and the 8500bp linear *XhoI* fragments, with selection for Leu+ colonies. Several integrants containing sets of the two expression cassettes present in the genome at various ratio were obtained. One transformant carrying four copies of CSV-S, and two copies of RTS (in addition to the five copies of S cassettes) was selected and given the official designation Y1845.

EXPRESSION OF THE RECOMBINANT PROTEIN:

Y1845 is grown, at 30°C, in **YNB** (Yeast Nitrogen Base available from Kracker Scientific Inc) minimal medium to an O.D.(620nm) of 0.5. Then cells are harvested and cellular extracts are prepared.

ANALYSIS OF EXPRESSION PRODUCTS BY IMMUNOBLOTTING:

EXTRACT PREPARATION:

Cells are re-suspended in Breaking Buffer and mechanically disrupted (glass beads). Extract is centrifuged for 5-10 minutes at 5000 rpm. Supernatant fraction is run on SDS – PAGE 12.5%.

Breaking Buffer:	50mM phosphate Na buffer(PH:7.5) 4mMEDTA Tween-20 0.5% + proteases inhibitor cocktail (Complete/ ROCHE)
Cell concentration:	100 ml culture (OD:0.5) in 5 ml breaking buffer = concentration of 10 OD unit /ml.
Crude extract clarification:	extract centrifuged 5-10 minutes/5000 rpm

DETECTION OF RECOMBINANT PROTEIN

Clarified extracts are run on SDS-PAGE 12.5%, proteins transferred to nitrocellulose membrane and subjected to immunostaining

Western blot analysis:

Reagent :	Mouse monoclonal antibody anti-S (prepared by GSK Biologicals)- (dilution: 1/500)

CsCl DENSITY GRADIENT CENTRIGUGATION:

The formation of particles in strain Y1845 was analyzed by CsCl density gradient centrifugation. Crude extracts (~20mg of total protein) was analyzed on a 12ml 1.5 M CsCl gradient (88 hours at 40.000 rpm, +8°C in a Beckman 70.1 Ti rotor). Fractions (~0.6ml) were collected and analyzed by immunoblot using an anti-S antibody. As shown in Figure 8 western blots peaks appear at the same fractions (No. 10 and 11) of the gradient corresponding to a buoyant density of 1.21 and 1.20 g/cm3. The triple particle formation is supported by gradient analysis.

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Claims,

1. An immunogenic protein particle comprising the following monomers:
   a. a fusion protein comprising sequences derived from a CS protein of *P.vivax* and the S antigen of Hepatitis B (CSV-S), and
   b. a fusion protein comprising sequences derived from CS protein of *P. falciparum* and S antigen of Hepatitis B (RTS), and
   c. optionally the S antigen derived from Hepatitis B.

2. A particle according to any preceding claim, wherein the Hepatitis B antigen is derived from an adw serotype.

3. A particle according to any preceding claim, which further comprises one or more further antigens derived from *P. falciparium* and/or *P. vivax.*

4. A particle according to any one of claims 1 to 4, wherein the *p. vivax* component is a hybrid polypeptide selected from DBP, PvTRAP, PvMSP2, PvMSP4, PvMSP5, PvMSP6, PvMSP7, PvMSP8, PvMSP9, PvAMA1 and RBP.

5. A particle according to any one of claims 1 to 4, which comprises the hybrid circumsporozoite protein sequence as shown in SEQ ID No 13.

6. A composition comprising a particle according to any one of the preceding claims and an adjuvant.

7. A composition according to claim 6, wherein the adjuvant is selected from the group comprising:

- metal salts such as aluminium hydroxide or aluminium phosphate,
- oil in water emulsions,
- toll like receptors agonist, (such as toll like receptor 2 agonist, toll like receptor 3 agonist, Toll like receptor 4 agonist, toll like receptor 7 agonist, toll like receptor 8 agonist and Toll like receptor 9 agonist),
- saponins, for example Quil A and its derivatives such as QS7 and/or QS21,
- CpG containing oligonucleotides,
- 3D –MPL,
- (2-deoxy-6-o-[2-deoxy-2-[(R)-3-dodecanoyloxytetra-decanoylamino]-4-o-phosphono-β-D-glucopyranosyl]-2-[(R)-3-hydroxytetradecanoylamino]-α-D-glucopyranosyldihydrogenphosphate),
- DP (3S, 9 R) –3-[(R)-dodecanoyloxytetradecanoylamino]-4-oxo-5-aza-9(R)-[(R)-3-hydroxytetradecanoylamino]decan-1,10-diol,1,10-bis(dihydrogenophosphate), and
- MP-Ac DP (3S-, 9R) -3-[(R) -dodecanoyloxytetradecanoylamino]-4-oxo-5-aza-9-[(R)-3-hydroxytetradecanoylamino]decan-1,10-diol,1 - dihydrogenophosphate 10-(6-aminohexanoate),

or combinations thereof.

8. A composition according to claim 6 or 7, wherein the adjuvant is selected from the group comprising:

- a saponin associated with a metallic salt, such as aluminium hydroxide or aluminium phosphate
- 3D MPL , QS21 and a CpG oligonucleotide, for example as an oil in water formulation,
- saponin in the form of a liposome, for example further comprise a sterol such as QS21 and sterol, and
- ISCOM,

9. A composition according to any one of claims 6 to 8, which further comprises one or more further antigens derived from *P. falciparium* and/or *P. vivax* in admixture.

10. A composition according to any one of claims 6 to 9, wherein the composition is a vaccine for parental use.

11. A particle as defined in any one of claims 1 to 5 or a composition as defined in any one of claims 6 to 10 for use in treatment.

12. Use of a protein as defined in any one of claims 1 to 5 or a composition according to any one of claims 6 to 10 for the manufacture of a medicament for the treatment/prevention of malaria.

13. A method of treating a patient susceptible to plasmodium infection comprising administering an effective amount of a protein as defined in any one of claims 1 to 5 or a composition as defined in any one of claims 6 to 11, particularly as a vaccine.

14. A host (vector/plasmid) encoding DNA sequences which are the components of a particle as defined in any one of claims 1-5 wherein said vector plasmid is suitable for inserting the DNA into a host for manufacturing the relevant particle or said plasmid is capable of, under appropriate conditions, manufacturing the components, which assemble into relevant particle.

15. A process for the production of a particle as defined in any one of claims 1 to 5, which process comprises expressing a nucleotide sequence encoding for said proteins in a suitable host and recovering the product.

16. A process according to claim 15 wherein the host is a yeast.

17. A process according to claim16, wherein the yeast is selected from the group comprising of:

Saccharomyces, Schizosaccharomycees, Kluveromyces, Pichia (eg Pichia pastoria), Hansenula, (eg *Hansenula polymorpha*), *Yarowia, Schwaniomyces, Schizosaccharomyces, Zygoaccharomyces,* such as *Saccharomyces cerevisiae, S. carlsberoensis, K. Lactis,* Y1834 and DC5

18. A process according to any one of claims 16 to 17, wherein product is recovered by lysis of the host cells by treatment with a suitable composition comprising a surfactant.

19. A process according to claim 18, wherein the surfactant is selected from the group comprising: Tween (such as Tween 20), briji, polyethylene and glycol.

20. A product obtainable by the process described in any one of claims 14 to 19.