ANTI-MALARIA VACCINE

The present invention relates to a novel use of a malaria antigen to immunise against malaria infection and disease. The invention relates in particular to the use of sporozoite antigens, in particular circumsporozoite (CS) protein or immunogenic fragments or derivatives thereof, combined with suitable adjuvants, to immunise malaria naïve individuals expecting to travel to endemic regions against malaria infection.

Malaria is one of the world's major health problems. During the 20th century, economic and social development, together with anti-malarial campaigns, have resulted in the eradication of malaria from large areas of the world, reducing the affected area of the earth's land surface from 50% to 27%. Nonetheless, given expected population growth it is projected that by 2010 half of the world's population, nearly 3.5 billion people, will be living in areas where malaria is transmitted (Hay, 2004) . Current estimates suggest that there are well in excess of 1 million deaths due to malaria every year, and the economic costs for Africa alone are staggering (Bremen, 2004).

These figures highlight the global malaria crisis and the challenges it poses to the international health community. The reasons for this crisis are multiple and range from the emergence of widespread resistance to available, affordable and previously highly effective drugs, to the breakdown and inadequacy of health systems and the lack of resources. Unless ways are found to control this disease, global efforts to improve health and child survival, reduce poverty, increase security and strengthen the most vulnerable societies will fail.

Malaria also poses risks to those traveling to or working in endemic regions who normally live in malaria free countries. The risks may be greater to this traveler population because they do not have any background immunity to malaria from natural exposure. Another aspect of the risk incurred by a traveler to a malaria endemic region is that the disease is often mis-diagnosed in its early stages due to the flu-like symptoms. When the severity increases and malaria is finally diagnosed, it can be too late. Within a

few days of the increased symptoms, death can result, for example, from cerebral malaria, or sometimes organ (e.g. liver or kidney) failure.

One of the most acute forms of the disease is caused by the protozoan parasite *Plasmodium falciparum* which is responsible for most of the mortality attributable to malaria. Another form of the disease is caused by *Plasmodium vivax*. *P. vivax* is the most widespread of all malarias. In addition to being present in tropical and sub-tropical regions, the ability of the parasite to complete its mosquito cycle at temperatures as low as 15 degrees Celsius, has allowed it to spread in temperate climates. However due to the fact that *P. vivax* infection is rarely fatal, the efforts to control *P. vivax* malaria (through vaccine development) are lagging behind those for *P. falciparum.*

An observation made 30 years ago provides strong support for the belief that an effective malaria vaccine will eventually be developed. Mice and humans can be protected against malaria by immunisation with live, radiation-attenuated malaria sporozoites. The persistence of intra-hepatic stage *in vivo* is required to produce and maintain protective immunity, but the underlying mechanisms have not yet been completely defined. Antibodies, CD8 and CD4 T-cells (Hoffman, 1996) have been implicated as critical effector immune mediators.

The life cycle of *Plasmodium sp (eg.P. falciparum or P. vivax)* is complex, requiring two hosts, man and mosquito for completion. The infection of man is initiated by the inoculation of sporozoites in the saliva of an infected mosquito. The sporozoites migrate to the liver and there infect hepatocytes (liver stage) 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.

The sporozoite stage of *Plasmodium sp (eg.P. falciparum or P. vivax)* has been identified as one potential target of a malaria vaccine. The major surface protein of the sporozoite is known as circumsporozoite protein (CS protein). This protein has been cloned, expressed and sequenced for a variety of strains, for example for *P. falciparum* the NF54 strain, clone 3D7 (Caspers, 1989). The protein from strain 3D7 is characterised by having a central immunodominant repeat region comprising a tetrapeptide Asn-Ala-Asn-Pro repeated 40 times but interspersed with four minor repeats of the tetrapeptide Asn-Val-Asp-Pro. In other strains the number of major and minor repeats varies as well as their relative position. This central portion is flanked by an N and C terminal portion composed of non-repetitive amino acid sequences designated as the repeatless portion of the CS protein.

GlaxoSmithKline Biologicals' RTS,S malaria vaccine based on CS protein has been under development since 1987 and is currently the most advanced malaria vaccine candidate being studied (Ballou, 2004). This vaccine specifically targets the pre-erythrocytic stage of *P. falciparum*.

RTS,S/AS02A (RTS,S plus adjuvant AS02A which contains immunostimulants QS21, 3D-MPL and an oil in water emulsion) was used in consecutive Phase I studies undertaken in The Gambia involving adults (Doherty, 1999), children aged 6-11 and 1-5 years (Bojang, 2005), which confirmed that the vaccine was safe, well-tolerated and immunogenic. Subsequently a paediatric vaccine dose was selected and studied in a Phase I study involving Mozambican children aged 1-4 years where it was found to be safe, well tolerated and immunogenic (Macete).

The RTS,S/AS02A vaccine has also shown evidence of efficacy in clinical trials in the USA and in the field in West Africa. RTS,S/AS02A induces significant IgG antibody responses to *P. falciparum* circumsporozoite protein and substantial T-cell immunity (Lalvani, 1999; Sun, 2003). Efficacy against *P.falciparum* experimental challenge in malaria-naïve volunteers in the USA has been estimated to be about 30-50% on average (Stoute, 1997; Stoute 1998; Kester, 2001) . The first of these studies (Stoute, 1997) was

86% effective in a small scale trial in which 6 out of 7 individuals immunized with RTS,S/AS02A were protected. Furthermore, a field study of semi-immune adults in The Gambia (preceeded by a safety study in Gambian adults (Doherty, 1999)) showed an overall efficacy of 34% over a period of one transmission season of 15 weeks, with 71% efficacy in the first nine weeks of follow-up and 0% efficacy thereafter (Bojang, 2001). These studies (Stoute, 1997; Stoute, 1998; Bojang, 2001; Kester, 2001) show efficacy against infection.

Results were recently reported from a trial using RTS,S/AS02A in young African children. It was discovered that the CS protein based RTS,S vaccine can confer not only protection against infection under natural exposure but also protection against a wide spectrum of clinical illness caused by *P. falciparum*. Children who received the RTS,S vaccine experienced fewer serious adverse events, hospitalisations, and severe complications from malaria, including death, than did those in the control group (Alonso, 2004).

Furthermore, the RTS,S vaccine efficacy against both new infections or clinical episodes appears either not to wane or to do so slowly. At the end of the 6 months follow up in the trial, the vaccine remained efficacious as there was a significant difference in the prevalence of infection. This is in contrast with previous trials in malaria naïve volunteers or Gambian adults which suggested that vaccine efficacy with RTS,S was short lived (Stoute, 1998; Bojang, 2001). Furthermore, after an additional follow-up period of 12 months, it was observed that the efficacy of the vaccine against an episode of clinical malaria did not significantly wane (Alonso, 2005).

Although the vaccine formulation described above shows clinical efficacy, additional improvements are still needed in order to increase both the number of individuals protected as well as the persistence of protection. New adjuvant formulations such as a formulation which contains QS21 and 3D-MPL in a liposome containing formulation (referred to herein as adjuvant B) have demonstrated a higher potency to boost T-cell immune response in various pre-clinical and clinical investigations.

In particular, what is needed for a vaccine for people who do not come from a malaria endemic region but are traveling for a limited period of time to regions where malaria is endemic, is better protection against *infection*. Clinical manifestation of malaria can only occur if there is a productive infection of the liver leading to the formation of merozoites and their release from the hepatic stage. These merezoites can then infect RBC and initiate the pathogenic blood stage of the parasite resulting in symptomatic clinical malaria. If there is no productive infection following exposure (i.e. no infection of the liver and/or no release of liver merozoites), then this is known as sterile immunity. A vaccine that would significantly reduce the risk of a productive liver infection, as defined above, following mosquito bites would be highly desirable for a traveler population that does not have pre-existing immunity, because by preventing the productive hepatic infection the vaccine would prevent any subsequent clinical manifestation. This can be contrasted with the aim of vaccine development targeting children or people in endemic regions, where the major aim would be to decrease the severity of disease and/or to decrease the number of episodes of disease, but not necessarily to prevent them completely. In theory, it would not be possible to indefinitely maintain sterile protection in people in endemic regions, and therefore they need to build up their own immunity by exposure to malaria infection. Furthermore, it may not be advisable to confer sterile protection on people living in endemic regions for an extended yet limited period of time.

We describe herein a challenge clinical trial consisting of a head to head comparison of RTS,S/AS02A versus RTS,S with a different adjuvant (adjuvant B) which contains QS21 and 3D-MPL in a formulation with cholesterol-containing liposomes, in a malaria naïve population (see Examples). Both T- and B-cell mediated immunity were investigated.

The results show that in a malaria naïve adult population the RTS,S antigen in combination with adjuvant B is greater than 50% more effective at protecting against productive hepatic infection following malaria challenge than RTS,S/AS02A. Thus, the RTS,S antigen in combination with adjuvant B is more effective in terms of the sterile protection which is required for malaria naïve individuals traveling to regions where

malaria is endemic. This increased efficacy conferred by adjuvant B is associated with an increased antigen specific immune response (antibodies and CD4-Th1 T-cells).

Therefore the present invention provides the use of a *Plasmodium* antigen or an immunogenic fragment or derivative thereof and an adjuvant comprising a lipid A derivative and a saponin in a liposome formulation, in the manufacture of a medicament for immunising travelers to endemic regions against productive malaria infection.

Generally, travelers to endemic regions will be malaria naïve. Thus, the invention applies to malaria naïve individuals.

The invention is particularly concerned with reducing the incidence of productive malaria infections in travelers to endemic regions, who may be any age but in particular adults.

A second aspect of the invention provides a formulation comprising a *Plasmodium* antigen or an immunogenic fragment or derivative thereof and an adjuvant, comprising a lipid A derivative and a saponin in a liposome formulation, for use in the immunisation of travelers to endemic regions against productive malaria infection.

A third aspect of the invention provides a method of prophylaxis of productive malaria infection in travelers to endemic regions comprising the administration of suitable amounts of a formulation comprising a *Plasmodium* antigen or an immunogenic fragment or derivative thereof and an adjuvant, comprising a lipid A derivative and a saponin in a liposome formulation.

In one embodiment of the invention the *Plasmodium* antigen is a *P. falciparum* antigen. In another embodiment of the invention the *Plasmodium* antigen is a *P. vivax* antigen.

Suitably the antigen is a pre-erythrocytic antigen.

The antigen may for example be selected from any antigen which is expressed on the sporozoite or other pre-erythrocytic stage of the parasite such as the liver stage. For example the antigen may be selected from circumsporozoite (CS) protein, liver stage antigen-1 (LSA-1) (see eg WO2004/044167), liver stage antigen-3 (LSA-3) (described e.g. in EP 0 570 489 and EP 0 833 917), Pfs 16kD (described in WO 91/18922 and EP 597 843), Exported antigen -1 (Exp-1) (described for example in Meraldi et al 2002, Parasite Immunol vol 24(3):141), sporozoite-threonine-asparagine-rich protein (STARP), sporozoite and liver stage antigen (SALSA), thrombospondin related anonymous protein (TRAP) (described in WO 90/01496, WO 91/11516 and WO 92/11868) and apical merezoite antigen-1 (AMA-1) (described in EP 0 372 019) which has recently been shown to be present at the liver stage (in addition to the erythrocytic stage). All of these antigens are well known in the field. The antigen may be the entire protein or an immunogenic fragment thereof or a derivative of either of these. Immunogenic fragments of malaria antigens are well know, for example the ectodomain from AMA-1 (described e.g. in WO 02/077195). Derivatives include for example fusions with other proteins which may be malaria proteins or non-malaria proteins such as HBsAg. Derivatives according to the invention are capable of raising an immune response against the native antigen.

The *Plasmodium* antigen may be fused to the surface antigen from hepatitis B (HBsAg).

One particular antigen for use in the invention is derived from the circumsporozoite (CS) protein and may be in the form of a hybrid protein with HBsAg. The antigen may be the entire CS protein or part thereof, including a fragment or fragments of the CS protein which fragments may be fused together.

The CS protein based antigen may be in the form of a hybrid protein comprising substantially all the C-terminal portion of the CS protein of *Plasmodium*, four or more tandem repeats of the CS protein immunodominant region, and the surface antigen from hepatitis B (HBsAg). The hybrid protein may comprise a sequence which contains at least 160 amino acids and which is substantially homologous to the C-terminal portion of

the CS protein. In particular "substantially all" the C terminal portion of the CS protein includes the C terminus devoid of the hydrophobic anchor sequence. Further, in the case of the antigen from *Plasmodium falciparum,* it contains 4 or more eg 10 or more Asn-Ala-Asn-Pro tetrapeptide repeat motifs. The CS protein may be devoid of the last 12 amino-acids from the C terminal.

The hybrid protein for use in the invention may be a protein which comprises a portion of the CS protein of *P. falciparum* substantially as corresponding to amino acids 207-395 of *P. falciparum* 3D7 clone, derived from the strain NF54 (Caspers, 1989) fused in frame via a linear linker to the N-terminal of HBsAg. The linker may comprise a portion of preS2 from HBsAg.

CS constructs for use in the present invention are as outlined in WO 93/10152. One particular construct is the hybrid protein known as RTS as described in WO 93/10152 (wherein it is denoted RTS*) and WO 98/05355, the whole contents of both of which are incorporated herein by reference.

A particular hybrid protein for use in the invention is the hybrid protein known as RTS which consists of:

- A methionine-residue, encoded by nucleotides 1059 to 1061, derived from the Sacchromyes cerevisiae TDH3 gene sequence. (Musti, 1983).

- Three amino acids, Met Ala Pro, derived from a nucleotide sequence (1062 to 1070) created by the cloning procedure used to construct the hybrid gene.

- A stretch of 189 amino acids, encoded by nucleotides 1071 to 1637 representing amino acids 207 to 395 of the circumsporozoite protein (CSP) of *Plasmodium falciparum* strain 3D7 (Caspers, 1989).

- An amino acid (Gly) encoded by nucleotides 1638 to 1640, created by the cloning procedure used to construct the hybrid gene.

- Four amino acids, Pro Val Thr Asn, encoded by nucleotides 1641 to 1652, and representing the four carboxy terminal residues of the hepatitis B virus (adw serotype) preS2 protein (Valenzuela, 1979).

- A stretch of 226 amino acids, encoded by nucleotides 1653 to 2330, and specifying the S protein of hepatitis B virus (adw serotype).

The RTS may be in the form of RTS,S mixed particles.

The RTS,S particles comprise two polypeptides RTS and S that may be synthesized simultaneously and spontaneously form composite particulate structures (RTS,S) e.g. during purification.

The RTS protein may be expressed in yeast, for example *S. cerevisiae*. In such a host, RTS will be expressed as lipoprotein particles. The recipient yeast strain may already carry in its genome several integrated copies of an hepatitis B S expression cassette. The resulting strain synthesizes therefore two polypeptides, S and RTS, that spontaneously co-assemble into mixed (RTS,S) lipoprotein particles. These particles may present the CSP sequences of the hybrid at their surface. The RTS and S in these mixed particles may be present at a particular ratio, for example 1:4.

The use of a further malaria antigen or fragment or derivative thereof in the invention is also encompassed within the invention. Other pre-erythrocytic antigens such as AMA-1, LSA-1, LSA-3 (described e.g. in EP 0 570 489 and EP 0 833 917) and Pfs 16kD, may be used in combination with RTS,S. Alternatively RTS,S may be used in combination with a blood stage antigen such as merezoite surface protein-1 (MSP-1) (described e.g. in US 4,837,016), erythrocyte binding antigen-175 (EBA-175) or MSP-3 (described e.g. in EP 0 666 916).

Immunogenic fragments of any of the antigens as described herein will contain at least one epitope of the antigen and display malaria antigenicity and are capable of raising an immune response when presented in a suitable construct, such as for example when fused to other malaria antigens or other non-malaria antigens, or presented on a carrier, the immune response being directed against the native antigen. Typically the immunogenic fragments contain at least 20, or at least 50, or at least 100 contiguous amino acids from the malaria antigen.

Derivatives of the antigens or fragments as described herein will similarly contain at least one epitope of the antigen and display malaria antigenicity and are capable of raising an immune response, the immune response being directed against the native antigen. Derivatives include for example fusions of the malaria antigen to another protein which may or may not be another malaria protein and may be, for example, HBsAg.

In accordance with the invention, an aqueous solution of the purified hybrid protein may be used directly and combined with the suitable adjuvant according to the invention. Alternatively, the protein can be lyophilized prior to mixing with the adjuvant. The adjuvant may be a liquid and is thus used to reconstitute the antigen into a liquid vaccine form.

Thus the invention further provides the use of a *Plasmodium* antigen or an immunogenic fragment or derivative thereof and an adjuvant comprising a lipid A derivative and a saponin in a liposome formulation, as described herein, in the manufacture of a kit for immunising travelers to endemic regions against malaria infection, wherein the antigen is provided in lyophilised form and the antigen and the adjuvant are mixed prior to administration.

The vaccine dose in accordance with the invention may be between 1-100 ug RTS,S per dose, for example 25 to 75 ug RTS,S, for example a dose of 50 ug RTS,S protein, which may be present in 500 ul (final liquid formulation). This is a suitable dose for use in

adults. A suitable dose for use in children is half the adult dose, that is 25 ug RTS,S, which may be present in 250 ul (final liquid formulation). Similar doses may be used for other antigens.

In accordance with the invention the antigen is combined with an adjuvant which comprises a lipid A derivative and a saponin in a liposome formulation.

Suitable adjuvants according to the invention are detoxified lipid A from any source and non-toxic derivatives of lipid A, which are preferential stimulators of a Th1 cell response (also herein called a Th1 type response).

An immune response may be broadly divided into two extreme categories, being a humoral or cell mediated immune response (traditionally characterised by antibody and cellular effector mechanisms of protection respectively). These categories of response have been termed Th1-type responses (cell-mediated response), and Th2-type immune responses (humoral response).

Extreme Th1-type immune responses may be characterised by the generation of antigen specific, haplotype restricted cytotoxic T lymphocytes, and natural killer cell responses. In mice Th1-type responses are often characterised by the generation of antibodies of the IgG2a subtype, whilst in the human these correspond to IgG1 type antibodies. Th2-type immune responses are characterised by the generation of a range of immunoglobulin isotypes including in mice IgG1.

It can be considered that the driving force behind the development of these two types of immune responses are cytokines. High levels of Th1-type cytokines tend to favour the induction of cell mediated immune responses to the given antigen, whilst high levels of Th2-type cytokines tend to favour the induction of humoral immune responses to the antigen.

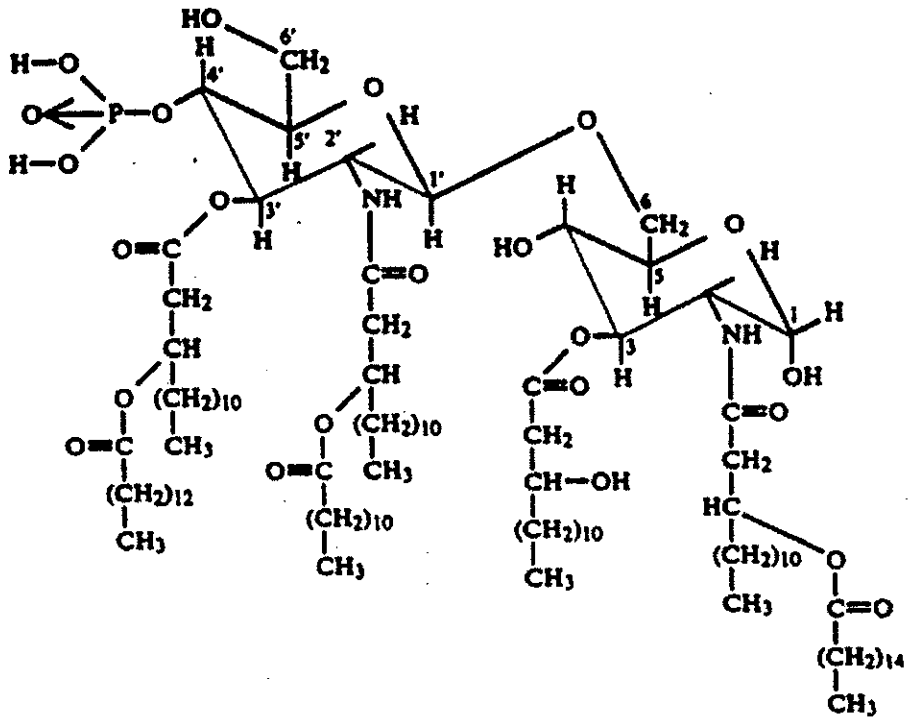
The distinction of Th1 and Th2-type immune responses is not absolute, and can take the form of a continuum between these two extremes. In reality an individual will support an immune response which is described as being predominantly Th1 or predominantly Th2. However, it is often convenient to consider the families of cytokines in terms of that described in murine CD4 +ve T cell clones by Mosmann and Coffman (Mosmann, 1989). Traditionally, Th1-type responses are associated with the production of the INF-gamma cytokines by T-lymphocytes. Other cytokines often directly associated with the induction of Th1-type immune responses are not produced by T-cells, such as IL-12. In contrast, Th2- type responses are associated with the secretion of IL-4, IL-5, IL-6, IL-10 and tumour necrosis factor-beta (TNF-beta).

It is known that certain vaccine adjuvants are particularly suited to the stimulation of either Th1 or Th2-type cytokine responses. Traditionally, indicators of the Th1:Th2 balance of the immune response after a vaccination or infection include direct measurement of the production of Th1 or Th2 cytokines by T lymphocytes *in vitro* after restimulation with antigen, and/or the measurement (at least in mice) of the IgG1:IgG2a ratio of antigen specific antibody responses.

Thus, a Th1-type adjuvant is one which stimulates isolated T-cell populations to produce high levels of Th1-type cytokines when re-stimulated with antigen *in vitro*, and induces antigen specific immunoglobulin responses associated with Th1-type isotype.

Adjuvants which are capable of preferential stimulation of the Th1 cell response are described in WO 94/00153 and WO 95/17209.

It has long been known that enterobacterial lipopolysaccharide (LPS) is a potent stimulator of the immune system, although its use in adjuvants has been curtailed by its toxic effects. A non-toxic derivative of LPS, monophosphoryl lipid A (MPL), produced by removal of the core carbohydrate group and the phosphate from the reducing-end glucosamine, has been described (Ribi, 1986) and has the following structure:



A further detoxified version of MPL results from the removal of the acyl chain from the 3-position of the disaccharide backbone, and is called 3-O-Deacylated monophosphoryl lipid A (3D-MPL). It can be purified and prepared by the methods taught in GB 2122204B, which reference also discloses the preparation of diphosphoryl lipid A, and 3-O-deacylated variants thereof.

A particular form of 3D-MPL for use in the present invention is in the form of an emulsion having a small particle size less than 0.2um in diameter, and its method of manufacture is disclosed in WO 94/21292. Aqueous formulations comprising monophosphoryl lipid A and a surfactant have been described in WO98/43670.

The bacterial lipopolysaccharide derived adjuvants to be used in the present invention may be purified and processed from bacterial sources, or alternatively they may be synthetic. For example, purified monophosphoryl lipid A is described in Ribi et al (Ribi, 1986), and 3-O-Deacylated monophosphoryl or diphosphoryl lipid A derived from *Salmonella sp.* is described in GB 2220211 and US 4912094. Other purified and synthetic lipopolysaccharides have been described (Hilgers, 1986; Hilgers, 1987; EP 0 549 074

B1). One particular bacterial lipopolysaccharide adjuvant for use in the invention is 3D-MPL.

Accordingly, the LPS derivatives that may be used in the present invention are those immunostimulants that are similar in structure to that of LPS or MPL or 3D-MPL. In another alternative the LPS derivatives may be an acylated monosaccharide, which is a sub-portion to the above structure of MPL.

Saponins are also Th1 immunostimulants. Saponins are well known adjuvants (Lacaille-Dubois, 1996). Suitable saponins for use in the invention include immunologically active saponins for example, Quil A (derived from the bark of the South American tree Quillaja Saponaria Molina), and immunologically active fractions thereof, are described in US 5,057,540 and "Saponins as vaccine adjuvants" (Kensil, 1996) and EP 0 362 279 B1. The haemolytic saponins QS21 and QS17 (HPLC purified fractions of Quil A) have been described as potent systemic adjuvants, and the method of their production is disclosed in US Patent No. 5,057,540 and EP 0 362 279 B1. Also described in these references is the use of QS7 (a non-haemolytic fraction of Quil-A) which acts as a potent adjuvant for systemic vaccines. Use of QS21 is further described in Kensil *et al.* (Kensil, 1991). Combinations of QS21 and polysorbate or cyclodextrin are also known (WO 99/10008). Particulate adjuvant systems comprising fractions of QuilA, such as QS21 and QS7 are described in WO 96/33739 and WO 96/11711.

The lipopolysaccharide and saponin immunostimulants described above for use in the invention are formulated together with a liposome carrier. For example, the carrier may comprise cholesterol containing liposomes as described in WO 96/33739.

Combinations of a monophosphoryl lipid A and a saponin derivative are described in WO 94/00153; WO 95/17210; WO 96/33739; WO 98/56414; WO 99/12565; WO 99/11241, and the combination of QS21 and 3D-MPL is disclosed in WO 94/00153.

Thus, suitable adjuvant systems for use in the invention include, for example, a combination of a monophosphoryl lipid A, such as 3D-MPL, together with a saponin derivative, particularly the combination of QS21 and 3D-MPL as disclosed in WO 94/00153. The adjuvant system includes a liposome carrier, for example cholesterol-containing liposomes, for example in a composition where the QS21 is quenched in cholesterol containing liposomes (DQ) as disclosed in WO 96/33739.

Thus the saponin such as QS21 may also be present in or associated with the membranes of the liposomes, as described in WO 96/33739. The 3D-MPL or other lipid A derivative may be present either entrapped in the membrane of the liposomes, or outside the liposomes, or both. One particular adjuvant for use in the invention comprises the two immunostimulants QS21 and 3D-MPL, in a formulation with cholesterol-containing liposomes, in which the 3D-MPL is entrapped within the liposomes and the QS21 is associated with the liposomes.

The amount of the protein 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 which specific adjuvant. Generally, it is expected that each dose will comprise 1-1000ug of protein, for example 1-200 ug, for example 10-100ug. An optimal amount for a particular vaccine can be ascertained by standard studies involving observation of antibody titres and other responses in subjects.

A suitable vaccination schedule for use with the invention is a primary course prior to travel to a malaria endemic region which may be completed for example at least 2-4 weeks prior arrival in the region. This primary course may involve between 1 and 3 doses, for example 2 or 3 doses, administered with an interval of at least 7 days, or between 1 and 4 weeks or for example a month between doses. A primary vaccination course may be followed by repeated boosts every six months for as long as a risk of infection exists. Periodic booster vaccinations may then be appropriate prior to repeat

travel to endemic regions. Suitable amounts of RTS,S protein per dose are as given herein above.

The vaccines of the invention may be administered by any of a variety of routes such as oral, topical, subcutaneous, mucosal (typically intravaginal), intraveneous, intramuscular, intranasal, sublingual, intradermal and via suppository.

The invention may be further used in a heterologous prime-boost regimen.

Instead of or in addition to repeat doses of the RTS,S or other polypeptide containing composition, a different form of the vaccine may be administered in a heterologous "prime-boost" vaccination regime. The priming composition and the boosting composition will have at least one antigen in common, although it is not necessarily an identical form of the antigen; it may be a different form of the same antigen.

Prime-boost immunisations according to the invention may be performed with a combination of protein and polynucleotide, particularly DNA-based formulations. Such a strategy is considered to be effective in inducing broad immune responses. Adjuvanted protein vaccines induce mainly antibodies and T helper immune responses, while delivery of DNA as a plasmid or a live vector induces strong cytotoxic T lymphocyte (CTL) responses. Thus, the combination of protein and DNA vaccination will provide for a wide variety of immune responses.

Thus the invention further provides the use of a *Plasmodium* antigen or an immunogenic fragment or derivative thereof and an adjuvant comprising a lipid A derivative and a saponin in a liposome formulation, as described herein, together with a polynucleotide encoding the *Plasmodium* antigen or an immunogenic fragment or derivative thereof, in the manufacture of a pharmaceutical kit for immunising travelers to endemic regions against productive malaria infection.

The invention also provides for a kit comprising a *Plasmodium* antigen or an immunogenic fragment or derivative thereof provided in lyophilised form, an adjuvant comprising a lipid A derivative and a saponin in a liposome formulation, and instructions specifying that the antigen, adjuvant, and optionally a further carrier, are to be mixed prior to administration to a traveler to an endemic region, thereby protecting said traveler against productive malaria infection.

Thus where RTS,S or another polypeptide based on CS protein is used as the polypeptide component of a prime-boost regimen, the polynucleotide component will encode CS protein or an immunogenic fragment or derivative thereof.

The DNA may be delivered as naked DNA such as plasmid DNA, or in the form of a recombinant live vector. Live vectors for use in the invention may be replication defective. Examples of live vectors which may be used are poxvirus vectors including modified poxvirus vectors, for example Modified Virus Ankara (MVA), alphavirus vectors for example Venezuelian Equine Encephalitis virus vectors, or adenovirus vectors for example a non-human adenovirus vector such as a chimpanzee adenovirus vector, or any other suitable live vector.

A suitable adenovirus for use as a live vector in a prime boost vaccine according to the invention is a low sero-prevalent human adenovirus such as Ad5 or Ad35 or a non-human originating adenovirus such as a non-human primate adenovirus such as a simian adenovirus. The vectors may be replication defective. Typically these viruses contain an E1 deletion and can be grown on cell lines that are transformed with an E1 gene. Suitable simian adenoviruses are viruses isolated from chimpanzee. In particular C68 (also known as Pan 9) (See US patent No 6083 716) and Pan 5, 6 and Pan 7 (W0 03/046124) may be used in the present invention. These vectors can be manipulated to insert a heterologous polynucleotide according to the invention such that the polypeptides according to the invention may be expressed. The use, formulation and manufacture of such recombinant adenoviral vectors is described in detail in WO 03/046142.

Protein antigens may be injected once or several times followed by one or more DNA administrations, or DNA may be used first for one or more administrations followed by one or more protein immunisations. It may be beneficial to administer DNA first, followed by protein.

Thus a particular example of prime-boost immunisation according to the invention involves priming with a single dose of a polynucleotide in the form of a recombinant live vector such as any of those described above, followed by boosting with one or more doses, for example 2 or 3 doses, of the adjuvanted protein such as RTS,S with an adjuvant described herein. The polynucleotide encodes the same protein (e.g. CS protein) or an immunogenic fragment or derivative thereof.

Thus the invention further provides a pharmaceutical kit comprising

a) a *Plasmodium* antigen or an immunogenic fragment or derivative thereof and an adjuvant comprising a lipid A derivative and a saponin in a liposome formulation, and

b) a polynucleotide encoding the *Plasmodium* antigen or an immunogenic fragment or derivative thereof;

wherein a) and b) are for use sequentially in any order, but particularly wherein b) is used as the prime and a) is used as the boost. The invention also provides for instructions with said kit, specifying that, in respect of a), the antigen, adjuvant, and optionally a further carrier, are to be mixed prior to administration to a traveler to an endemic region.

The composition a) may be any polypeptide composition as described herein, in a suitable adjuvant as described herein. For example a) may be a composition comprising RTS,S and an adjuvant comprising QS21 and 3D-MPL in a liposome formulation, and b) may be a live vector as described herein such as an adenovirus vector e.g. a chimpanzee adenovirus vector, encoding CS protein or an immunogenic fragment or derivative thereof.

Both the priming composition and the boosting composition may be delivered in more than one dose. Furthermore the initial priming and boosting doses may be followed up with further doses which may be alternated to result in e.g. a DNA prime / protein boost / further DNA dose / further protein dose.

Appropriate pharmaceutically acceptable diluents or excipients for use in the invention are well known in the art and include for example water or buffers. Vaccine preparation is generally described (Powell, 1995; Voller, 1978). Encapsulation within liposomes is described, for example, by Fullerton, U.S. Patent 4,235,877. Conjugation of proteins to macromolecules is disclosed, for example, by Likhite, U.S. Patent 4,372,945 and by Armor et al., U.S. Patent 4,474,757.

In another aspect the invention provides a method for determining whether an individual is protected against malaria following administration of a malaria antigen composition, in particular a pre-erythrocytic malaria antigen composition, to the individual, which method comprises measuring the level of CD4 T cells raised in the individual specific for the malaria antigen. Also there is provided a method for determining whether an individual is protected against malaria following administration of a malaria antigen composition, in particular a pre-erythrocytic malaria antigen composition, to the individual, which method comprises measuring the concentration of antibodies raised in the individual specific for the malaria antigen.

In a further aspect the invention provides a method for assessing the efficacy of a candidate vaccine, particularly a pre-erythrocytic candidate vaccine, in the prevention of malaria, which method comprises measuring the level of CD4 cells raised in an individual against the candidate vaccine.. Also there is provided a method for assessing the efficacy of a candidate vaccine, particularly a pre-erythrocytic candidate vaccine, in the prevention of malaria, which method comprises measuring the concentration of specific antibodies raised in an individual against the candidate vaccine. In a more specific embodiment this vaccine comprises a *Plasmodium* antigen or an immunogenic

fragment or derivative thereof and an adjuvant comprising a lipid A derivative and a saponin in a liposome formulation

Examples

Example 1: Vaccination using RTS,S and Adjuvant B and experimental malaria challenge.

The Vaccines

RTS,S: RTS is a 51 kDa hybrid polypeptide chain of 424 amino acids (a.a.), consisting of 189 amino acids derived from a sporozoite surface antigen (the CS protein central tandem repeat and carboxyl-terminal regions, 189 amino acids in total) of the malaria parasite *P. falciparum* strain NF54 (the CSP antigen, a.a. 207 to 395), fused to the amino terminal end of the hepatitis B virus S protein. S is a 24 kDa polypeptide (226 amino acids long) corresponding to the surface antigen of hepatitis B virus (HBsAg), and is the antigen used in the GSK Biologicals Engerix-B® vaccine.

The two proteins are produced intracellularly in yeast (*S. cerevisiae)* and spontaneously assemble into mixed polymeric particulate structures that are each estimated to contain approximately 100 polypeptides.

The preparation of RTS,S is described in WO 93/10152.

A full dose of RTS,S/AS02A (GlaxoSmithKline Biologicals, Rixensart, Belgium) contains 50 ug of lyophilised RTS,S antigen reconstituted in 500 uL of AS02A adjuvant - oil in water emulsion containing the immunostimulants 3D-MPL® (GlaxoSmithKline Biologicals, Montana, USA) and QS21, 50 ug of each.

A full dose of RTS,S/Adjuvant B (GlaxoSmithKline Biologicals, Rixensart, Belgium) contains 50ug of lyophilised RTS,S antigen reconstituted in 500 uL of adjuvant B

containing the immunostimulants 3D-MPL® and QS21 (50 ug of each) in a formulation with cholesterol-containing liposomes. The liposomes can be prepared from a mixture of de-oleic phosphatidylcholine (DOPC), cholesterol and 3D-MPL in organic solvent, wherein the mixture is dried down. An aqueous solution is then added to suspend the lipid. The suspension is microfluidised until the liposome size is reduced to be sterile filterable through a 0.2 um filter. Typically the cholesterol: phosphatidylcholine ratio is 1:4 (w/w), and the aqueous solution is added to give a final cholesterol concentration of 5 to 50 mg/ml. QS21 is added to the cholesterol-containing liposomes.

Methodology

This clinical trial has evaluated the safety, reactogenicity, immunogenicity and preliminary efficacy of a malaria vaccine containing the antigen RTS,S adjuvanted with either AS02A or adjuvant B.

103 subjects were recruited into two cohorts and were randomized to receive 3 doses of either vaccine according to a 0, 1, 2 month vaccination schedule. Because of the large numbers of subjects involved, the cohorts were recruited and challenged sequentially.

For each cohort, volunteers were requested to undergo a standardised primary malaria challenge (Chulay, 1986) two to four weeks following third dose. The primary challenge involved allowing five *P.falciparum* sporozoite infected *Anophelese stevensi* mosquitos to feed on each challenge volunteer for a period of five minutes. For each cohort, twelve unvaccinated control volunteers were also challenged.

Approximately six months after the primary challenge, volunteers who were protected at the primary challenge were asked to undergo a repeat challenge. No additional doses of vaccine were administered between challenges. The repeat challenge was carried out as for the primary challenge. For each cohort, six unvaccinated control volunteers were also challenged.

After each challenge subjects were followed daily for a period of at least 30 days to assess whether they had become infected with malaria. The principle method of detecting infection was an evaluation of a Giesma-stained peripheral blood smear to detect asexual stage parasites by light microscopy. This indicates that a subject has undergone a productive infection, with parasites having been released from the liver and progressed to erythrocytic stage. Thus sterile protection against challenge has not been achieved. At the first sign of infection subjects were declared to be positive for malaria and received a curative dose of chloroquine. The primary efficacy readout was sterile protection, that is the subject never developed asexual stage parasitaemia. In addition the time between the challenge and the appearance of parasitaemia in those that were not fully protected was recorded.

In addition, peripheral blood mononuclear cell (PBMC) samples were collected at pre-vaccination, at 2-weeks post II and at 14-28 days post III vaccination (Day of challenge: DOC).

PBMCs samples were used to evaluate CD4 and CD8 T-cell responses by cytokine flow cytometry. The latter technology allows the quantification of T-cells specific to a given antigen. Antigen-specific CD4 and CD8 T cells were enumerated by flow cytometry following conventional immunofluorescence labeling of cellular phenotype as well as intracellular cytokines production. Briefly, peripheral blood antigen-specific CD4 and CD8 T cells can be restimulated in vitro to produce IL-2, CD40L, TNF-alpha or IFN-gamma when incubated with their corresponding antigen. Both HBs (hepatitis B surface antigen) and CSP pools of peptide were used as antigens to restimulate antigen-specific T cells. Results were expressed as a frequency of CD4 or CD8 T-cells expressing at least two different cytokines among CD40L, IL-2, TNF-alpha, or IFN-gamma within the CD4 or CD8 T cell sub-population.

Antibody levels are determined by evaluating antibody (IgG) responses to the *P. falciparum* CS-repeat region as measured using standard ELISA methodologies with the recombinant protein R32LR as capture antigen. Briefly, the R32LR protein

[corresponding to the repeated region (NANP) of the Plasmodium falciparum circumsporozoite protein (CSP)] is coated onto a 96-well plates. After saturation of the plates, the serum samples serial dilutions are added directly to the plates. Antibodies to R32LR present in the serum sample will bind to the pre-coated R32LR. The plates are washed. A peroxidase labeled Goat anti-Human IgG(γ)antibody is added, and it will bind to anti-CS IgG antibodies. After another washing step, the addition of a chromogen substrate solution specific for the peroxidase provides a mean of detecting anti-CS IgG bound to the pre-coated antigen. The peroxidase catalyses a color reaction. The intensity of the color formed is proportional to the titre of the anti-CS IgG antibodies contained in the serum. Anti-repeat antibody levels are determined relative to a known serum standard run on each plate, and are expressed in μg/ml.

Results

Cohort 1:

Adjuvant	Number of volunteers vaccinated	Number of volunteers challenged (number protected)	Number of volunteers rechallenged (number protected)
Adjuvant B	26	17 (10)	5 (3)
AS02A	25	24 (9)	5 (1)
TOTAL:	51	41	10

Cohort 2:

Adjuvant	Number of volunteers vaccinated	Number of volunteers challenged (number protected)	Number of volunteers rechallenged (number protected)
Adjuvant B	Figure not available	19 (8)	Not done
AS02A	Figure not available	20 (5)	Not done
TOTAL:	52	39	

Cohorts 1 & 2 combined for primary challenge:

Adjuvant	Number of volunteers vaccinated and challenged (primary challenge)	Number of volunteers protected	Number of volunteers infected	Vaccine efficacy
Adjuvant B	36	18	18	50%
AS02A	44	14	30	32%

Thus in this trial, adjuvant B was found to be more effective at protecting naïve individuals against malaria infection. 50% of individuals challenged from the adjuvant B group were protected compared to 32% of individuals challenged from the AS02A group. This represents an improvement in protection between the two adjuvants of greater than 50%.

Furthermore, in cohort 1, adjuvant B was found to be significantly more potent than AS02A to induce CD4 T-cell responses directed against antigens present in RTS,S (Figure 1. p=0.01663). The combined data for cohorts 1 and 2 was less significant (Figure 2. p=0.07).

Before vaccination, there were no detectable CD4/CD8 T-cell responses directed against HBs or CSP. In contrast, at 2-week post II vaccination as well as 2-week post III vaccination (DOC: day of challenge), HBs- and CSP-specific CD4 T-cells were detected in most individuals vaccinated with both formulations. At the same time points, no detectable CD8 T-cell response was observed. These observations demonstrate that the assay used for T-cell immuno-monitoring is specific and sensitive, which is a prerequisite to perform formal comparison between groups having received different vaccine formulations.

Figure 1 shows that individuals from cohort 1 vaccinated with RTS,S/adjuvant B have a higher frequency of CSP-specific CD4 T-cells compared to those vaccinated with RTS,S/AS02A both at 2-week post II and post III vaccination (DOC). A similar conclusion can be drawn for HBs-specific CD4 T-cells producing IFN-gamma and

another cytokine among IL-2, CD40L, TNF-alpha at 2-week post II vaccination (data not shown).

Figure 2 shows the same study as Figure 1, except that it incorporates the data from both cohorts.

In Figures 1 & 2, results are expressed as a frequency of CD4 T-cells expressing at least two different cytokines among CD40L, IL-2, TNF-alpha, or IFN-gamma within 10^6 CD4 T-cells.

A similar picture can be seen in terms of anti-CSP specific antibody responses. From **Figure 5** it can be seen that the concentration of antibodies raised in response to RTS,S/Adjuvant B was significantly greater than the concentration raised in response to RTS,S/AS02A (see particularly DOC - 2 weeks post III vaccination ($P = 0.00793$), but an effect is noticeable 2 weeks post II vaccination). Results are expressed as geometric mean concentration (GMC). Pre refers to the initial time point, before any doses are administered.
M1 refers to the time point 2 weeks after the first dose.
M2 refers to the time point 2 weeks after the second dose.
DOC refers to the 'Date of Challenge', which is 2 weeks after the third dose.

Protection against malaria challenge is associated with a significant higher CD4 T-cell response and specific antibody response to CSP.

Increased immunogenicity of RTS,S/adjuvant B compared to RTS,S/AS02A does not necessary imply that it will translate into a biologically relevant effect. However, individuals vaccinated with RTS,S/adjuvant B in this trial have shown increased level of protection (18 out of 36 individuals: 50%) against malaria challenge compared to those vaccinated with RTS,S/AS02A (14 out of 44 individuals: 32%). A possible link between amplitude of CD4 T-cell response and protection to malaria has been therefore found.

If the above hypothesis is true, protected individuals should have a higher CD4 T-cell response than individuals vaccinated with RTS,S/AS02A or even RTS,S/adjuvant B. **Figures 3 and 4**, for the 1st cohort and the combined cohorts respectively (with both adjuvant groups pooled), clearly confirm the above hypothesis and support the idea that CSP-specific CD4 T-cells play a significant role in protection. Consistently, statistical analysis made on samples collected at 2-week post II as well as 2-week post III vaccination demonstrate that the difference in frequency between protected and non-protected individuals is statistically significant.

In Figures 3 and 4 results are expressed as a frequency of CD4 T-cells expressing at least two different cytokines among CD40L, IL-2, TNF-alpha, or IFN-gamma within 10^6 CD4 T-cells. Immunogenicity analysis also indicates that, in contrast to CSP-specific CD4 T-cells, HBs-specific CD4 T-cells are not associated with protection against malaria at 2-week post II as well as 2-week post III vaccination ($p=0.14$ and $p=0.053$, receptively). This further consolidates the relevance of the above results and strongly suggests that protection is specifically linked with the presence of CD4 T-cells capable of recognizing CSP but not HBs peptides.

Finally, since there is no detectable CD8 T-cell response, it can also be concluded that, most likely, malaria pre-erythrocytic stage protection conferred by RTS,S adjuvanted with adjuvant B or AS02A is not likely due to induction of CSP-specific CD8 T-cells following vaccination.

A similar picture is observed from monitoring anti-CSP antibody responses. **Figure 6** shows antibody concentrations in protected and unprotected individuals (results relate to both cohorts with both adjuvant groups pooled).
It is clear that those individuals who are protected show a significantly higher antibody concentration than those who are not protected ($P < 0.0001$).

Discussion

The above results clearly demonstrate that an association between CSP-specific CD4 T-cell and antibody responses on the one hand and protective status on the other hand against malaria challenge exists. The mechanism by which CSP-specific CD4 T-cells or antibodies would exert an anti-parasitic effect is not known. However, analysis also clearly identified a minority of individuals having a high CD4 T-cell or high antibody response that are not protected. This means that strong CD4 T-cell response or high antibody response to CSP do not alone predict protection against malaria challenge.

Different technologies have been developed to monitor T-cell responses such as lymphoproloferation, cytokine secretion, tetramer staining, elipsot or cytokine flow cytometry. The latter has been recently selected as the lead technology on the basis of excellent repeatability/reproducibility data as well as relevant marker detection (CD4, CD8, CD40L, IL-2, TNF, IFNg). A specific analytical methodology has also been identified, which resolves the high background issue often seen with cytokine flow cytometry approaches. The present report demonstrates the feasibility of using cytokine flow cytometry for robust monitoring of T-cell responses in a human clinical trial. Furthermore, it also demonstrates that it is possible to identify a marker of protection that is not directly linked to humoral immunity.

Although adjuvant B has been demonstrated to be significantly more potent than AS02A formulation to induce CSP-specific CD4 T-cells and antibodies, the difference in terms of frequency of CD4 T-cells and antibody concentrations is relatively modest and one could have concluded that it might not be relevant biologically. Data obtained in this clinical trial allow formal assessment of the biological relevance of such differences using protection against malaria data as a biologically relevant marker. Analysis clearly indicates that modest but significant differences between adjuvant in terms of T-cell frequencies and antibody concentrations translate into significantly higher degree of protection against malaria challenge.

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Claims

1. The use of a *Plasmodium* antigen or an immunogenic fragment or derivative thereof and an adjuvant comprising a lipid A derivative and a saponin in a liposome formulation, in the manufacture of a medicament for immunising travelers to endemic regions against productive malaria infection, wherein the *Plasmodium* antigen is the circumsporozoite (CS) protein or an immunogenic fragment or derivative thereof capable of raising an immune response against *Plasmodium*.

2. The use according to claim 1 wherein the CS protein or fragment is fused to the surface antigen from hepatitis B (HBsAg).

3. The use according to claim 2 wherein the CS protein or fragment is in the form of a hybrid protein comprising substantially all the C-terminal portion of the CS protein of *Plasmodium*, four or more tandem repeats of the CS protein immunodominant region, and the surface antigen from hepatitis B (HBsAg).

4. The use according to any one of claims 1 to 3, wherein the hybrid protein comprises a sequence of CS protein of *P.falciparum* substantially as corresponding to amino acids 207-395 of *P.falciparum* NF54 strain 3D7 clone CS protein fused in frame via a linear linker to the N-terminal of HBsAg.

5. The use according to claim 4 wherein the hybrid protein is RTS.

6. The use according to claim 5 wherein the RTS is in the form of mixed particles RTS,S.

7. The use according to claim 6 wherein the amount of RTS,S is 25 or 50 ug per dose.

8. The use according to any one of claims 1 to 7 wherein the adjuvant comprises 3D-MPL and QS21.

9. The use according to claim 8 wherein the QS21 is quenched in cholesterol containing liposomes.

10. The use according to any one of claims 1-8, in conjunction with a polynucleotide encoding the *Plasmodium* antigen or an immunogenic fragment or derivative thereof, wherein the antigen/adjuvant mixture and the polynucleotide are for use sequentially in any order.

11. The use according to claim 10, wherein the polynucleotide is used first.

12. The use of a *Plasmodium* antigen or an immunogenic fragment or derivative thereof and an adjuvant comprising a lipid A derivative and a saponin in a liposome formulation, in the manufacture of a kit for immunising travelers to endemic regions against malaria infection, wherein the antigen is provided in lyophilised form and the antigen and the adjuvant are mixed prior to administration and wherein the *Plasmodium* antigen is the circumsporozoite (CS) protein or an immunogenic fragment or derivative thereof capable of raising an immune response against *Plasmodium*.

VB61495 seq list

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