1 CHAPTER ONE:

LITERATURE REVIEW

1.1 Definition

Malaria is both an acute and chronic disease caused by protozoa of the genus *Plasmodium*. Four species cause human malaria namely, *P. falciparum, P. vivax, P. ovale* and *P. malariae*. The protozoa are transmitted to humans by female mosquitoes of the *Anopheles* genus. The parasites asexual stage cycles are spent in the human erythrocyte. *P. falciparum* is the most common to infect humans and causes the most fatalities (Virtual Naval Hospital, 1998).

1.2 Historical review

Malaria is a very old disease and prehistoric man is thought to have suffered from it. Malaria probably originated in Africa and accompanied human migration to the Mediterranean shores, India and South East Asia. In the past it used to be common in the marshy areas around Rome and the name is derived from the Italian word "malaria" or "bad air". It was also known as Roman fever. The association with stagnant waters led the Romans to begin drainage programs, the first action against malaria (Malaria: an Online Resource, 2001; Bradley, 1996).

The malaria parasite was first detected in fresh blood drawn from patients in the 1880s and in 1889 Laveran, working in Algeria, deduced the protozoal cause of malaria. It wasn't until 1897 that the *Anopheles* mosquito was discovered as the vector for the disease. The study of the parasite was hindered by the staining method until in 1892 when an improved method of staining blood smears was developed, and the morphology of the parasite could be studied. This led to the identification of different stages such as the ring forms, trophozoites, schizonts and merozoites. Detail was however unobtainable through the use of light microscopy, and only until the

invention of the electron microscope in 1932 was the study of biological material possible. The electron microscope was first applied to the field of malariology in the study of *Plasmodium vivax* in 1942. Only in 1956 was it established that the parasite was within the host erythrocyte. In 1957 the fine structure of the parasite was eventually studied and various organelles were identified, including a nucleus, mitochondrion, a well-developed endoplasmic reticulum, pigment granules, lipid bodies and a plasma membrane surrounding the parasite (Aikawa, 1971).

The earliest treatment of malaria dates back to 1600 in Peru where the native Peruvian Indians used the bitter bark of the cinchona tree (which contained quinine) to treat the disease. By 1649 the bark was available in England and was known as 'Jesuits powder'. The first radical step towards the annihilation of malaria was in 1944 when DDT made its first debut in Italy after its discovery as an insecticide two years earlier (Bradley, 1996). This initial step made the ideal of global eradication of the malaria vector, *Anopheles*, seem possible and widespread systematic control measures such as DDT spraying, coating marshes with paraffin, draining stagnant water and the use of nets and cheap drugs were implemented (Bradley, 1996).

Incredibly this global eradication policy did not include Africa where the majority of malarial disease is manifest as it had insufficient infrastructure to support the policy. The policy was abandoned in 1969 but countries such as Hungary, Bulgaria, Romania, Yugoslavia, Spain, Italy, Netherlands and Portugal had managed to eradicate their epidemic malaria. The disease is currently epidemic in 91 countries. In 1989 the World Health Organisation (WHO) declared malaria control to be a global priority due to the worsening situation, and in 1993 the urge to increase control measures was made. Today some 500 million people in Africa, India, South East Asia and South America are exposed to endemic malaria and it is estimated to cause 2-4 million deaths annually, one million of which are children (Bradley, 1996).

1.3 The asexual blood stages

The malaria parasite enters into the asexual cycle development stage in the human host. The sporozoites are injected from the mosquito salivary glands into the human

host as the mosquito must inject anti-coagulants to ensure an even-flowing meal. Once in the blood stream the sporozoites travel to the liver and penetrate hepatocytes where they remain for 9-16 days and multiply (Figure 1.1; Sharma, 2000). The hepatocytes rupture to release merozoites into the blood stream for invasion of red blood cells. The invading merozoite appears to be attached to the invaded red blood cell by filamentous material leading from apical organelles at one end. These filaments are cleaved upon entry into the host cell (Howard and Schmidt, 1995; Bannister *et al*, 2000). Apical organelles (rhoptries, micromenes and dense granules) discharge their contents during invasion that change the shape and composition of the invaded red blood cell membrane (RBCM) and assist in erythrocyte invasion. These proteins induce the erythrocyte membrane (EM) to form an invasion pit that eventually envelops the merozoite (Bannister *et al*, 2000).

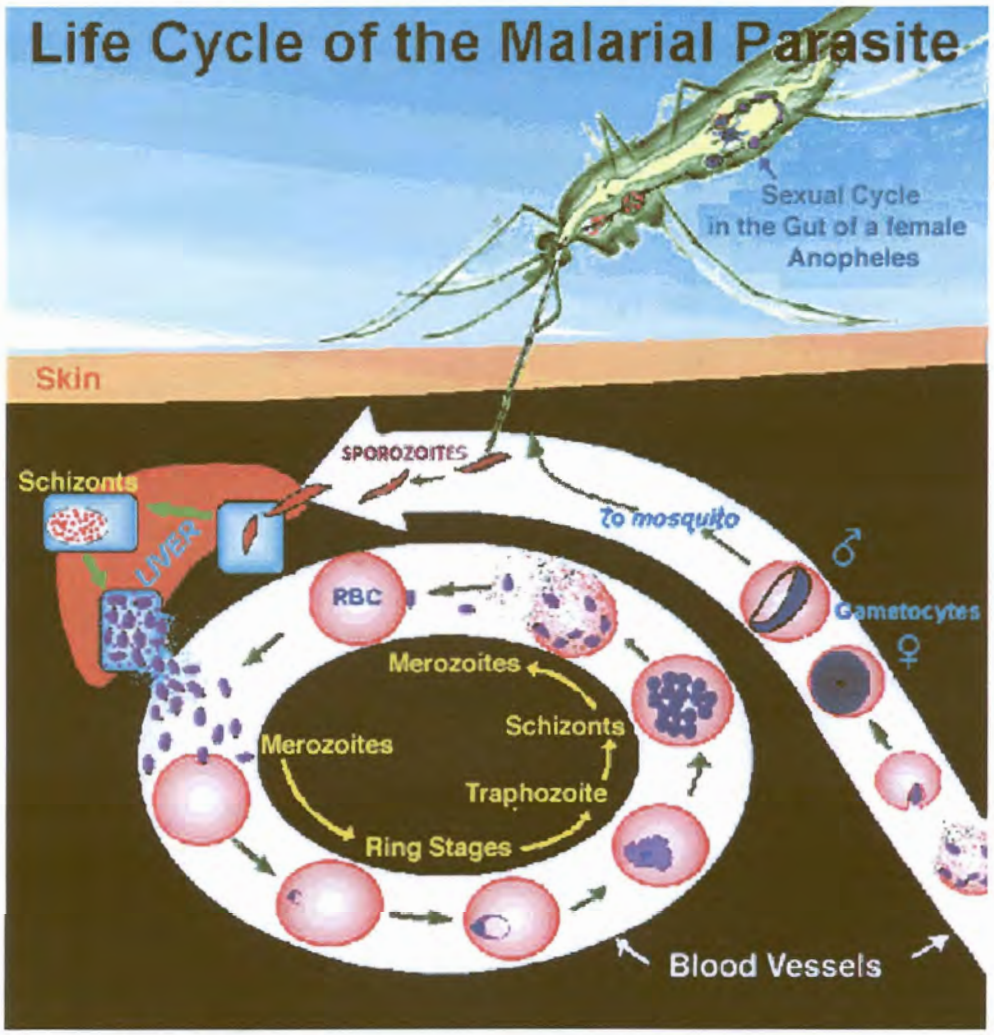

Figure 1.1: Schematic representation of the asexual cycle of *Plasmodium falciparum* in the human host (Sharma, 2000).

The subsequent ring stage parasites are surrounded by a membrane called the parasite vacuole membrane (PVM), which is probably derived from the erythrocyte membrane but is devoid of erythrocyte proteins and cytoskeleton (Howard and Schmidt, 1995; Elford *et al*, 1995). For the next 24 hours the parasite develops organelles such as the tubovesicular membrane (TVM) in the cytoplasm of the infected erythrocyte and a food vacuole within the parasite (Langreth *et al*, 1978).

The surface morphology of the infected erythrocyte is dramatically changed from 10-30 hours post-invasion during the trophozoite stage. From 24-36 hours after invasion the trophozoite matures to a schizont and occupies 80-90% of the volume of the infected erythrocyte. Schizogony (36-48 hours) is the final stage when the parasite engages in mitosis and development in the cytoplasm of secretory structures of the apical complex is prominent. 48 hours after invasion the daughter merozoites are fully developed and are released by rupture of the erythrocyte membrane and parasite vacuole membrane (Langreth *et al*, 1978).

1.4 Pathogenesis of malaria

Clinical symptoms and signs of malaria occur shortly before or at the time of red blood cell lysis. Symptoms include fever caused by the release of merozoites, malaria pigments, parasite proteins and cellular debris (Virtual Naval Hospital, 1998). This results in the host's release of pro-inflammatory cytokines, such as tumour necrosis factor (TNF), which triggers the onset of pathology (Clark and Scholfield, 2000). Patients infected with one of the three less severe human malarias, *P. ovale*; *P. malariae* or *P. vivax* show symptoms of chills followed by high fever that re-occur in a continuous cycle. This is not symptomatic of *P. falciparum*, which produces continual fever with irregular temperature spikes (Virtual Naval Hospital, 1998).

Nearly all of the deaths from malaria in African children are due to *P. falciparum* (Brown *et al*, 2000). It is the only type that causes microvascular disease and tissue damage. Morbidity is usually associated with cerebral malaria, which is symptomatic of seizures and impaired consciousness. Cerebral malaria is caused by microvascular obstruction (cytoadherence and sequestration) that prevents the exchange of glucose

and oxygen at the capillary level and causes hypoglycaemia, lactic acidosis, and fever. This results in impaired brain function but does not cause tissue damage since patients who are given prompt treatment can recover fully (Virtual Naval Hospital, 1998).

Cytoadherence is mediated by a family of antigenic variant (*var*) proteins collectively known as *var*/ PfEMP1 (*P. falciparum* erythrocyte membrane protein; Deitsch and Wellems, 1996). These proteins are associated with knob protrusions that have been implicated in cytoadherence (Cooke *et al*, 2000). Other proteins included in knobs are the knob associated histidine rich protein (KAHRP) and PfEMP3 (Cooke *et al*, 2000; Deitsch and Wellems, 1996). These knobs adhere to endothelial cells or placental syncytiotrophoblasts of the host. Two receptors on the host cell, namely CD36 and ICAM-1 (cluster determinant 36 and intercellular adhesion molecule-1, respectively), have been implicated in cytoadhesion, but recent studies with polymorphisms in ICAM-1 (Fernandez-Reyes *et al*, 1997) and mutations in CD36 (Aitman *et al*, 2000) have challenged the involvement of these receptors in infected red blood cells (IRBC) cytoadherence.

A prognosis of cerebral malaria is a very high plasma concentration of the pro-inflammatory cytokines TNF. A key role has been suggested for the *P. falciparum* toxin glycosylphosphatidylinositol (GPI) released upon rupture of mature schizonts, which appears to be a necessary and sufficient trigger for TNF release in tissues. GPI has now been implicated as the key toxin involved in malaria pathogenesis (Brown *et al*, 2000; Cooke and Cowman, 2000). Side effects similar to those of malaria pathogenesis are observed in tumour patients administered TNF-α. A correlation has been made between TNF-α and interleukin-10 (IL-10) by *in vitro* studies. IL-10 may be produced soon after the production of TNF-α to regulate the inflammatory activities of TNF-α. TNF-α induces fever and elevates body temperature, which can initially help to suppress parasitemia. But prolonged exposure to TNF-α promotes severe disease (Othoro *et al*, 1999). Studies have also shown that TNF-α suppresses erythropoiesis (production of red blood cells) contributing to anaemia development (Clark and Chaudhri, 1988). Therefore persons that produce IL-10 in balanced levels to regulate TNF-α may not develop severe or moderate malaria anaemia (Othoro *et al*, 1999).

T cell subsets and their products are essential in regulating both antibody formation and antibody independent protection. The $CD4^+$ T cell subset is thought to help with blood stage immunity, whilst $CD8^+$ T cells are known for their activity against liver stage parasite infection (Winkler *et al*, 1999). Immunisation of mice (Nussenzweig *et al*, 1967), monkeys (Bianco *et al*, 1970) and humans (Clyde *et al*, 1973) with irradiated sporozoites protected against challenge with live sporozoites, but *in vivo* depletion of $CD8^+$ T cells reduced protection (Weiss *et al*, 1988). Treatment of irradiated sporozoite immunised mice with monoclonal antibodies against gamma interferon (IFN-γ) or with an inhibitor of inducible nitric oxide synthetase also eliminated protection (Schofield *et al*, 1987; Seguin *et al*, 1994). Vaccine development has focused on inducing antibodies against the sporozoite surface to elicit a $CD8^+$ T cells response. Now new data indicates the involvement of $CD4^+$ T cells in irradiated sporozoite immunised mice. Protection against sporozoite challenge was eliminated with treatment of these immunised mice with both anti-$CD4^+$ and anti-$CD8^+$ antibodies (Renia *et al*, 1991; reviewed in Oliveira-Ferreira and Daniel-Ribeiro, 2001).

Another factor induced by the release of cytokines during *P. falciparum* infection is the generation of an inducible nitric oxide synthase (iNOS), which in turn produces a continual supply of nitric oxide (NO; Clark and Schofield, 2000). This short-lived molecule permeates freely through membranes and is responsible for a diverse array of unrelated but essential physiological functions in organs (Clark and Cowden, 1999). NO released in high concentrations under the influence of TNF may produce "nonsense signals" to the brain, which is thought to be a contributing factor towards cerebral malaria pathology (Clark *et al*, 1991). The iNOS is also induced by hypoxia (low oxygen levels in the blood), which is a consequence of infected erythrocyte sequestration (Clark and Cowden, 1999). Hypoxia also acts through the transcription factor hypoxia-inducible factor-1 (HIF-1) to increases other glycolytic enzymes and transport factors including the human glucose transporter GLUT1 required to accelerate glycolysis, which could indicate a common role for NO (Clark and Cowden, 1999). The role of NO production has been debated and its mode of action is not yet known (Brown *et al*, 2000)

Other symptoms of *P. falciparum* infection include renal failure resulting from renal tubules becoming clogged with haemoglobin and released malaria pigment, which causes glucose deprivation at the renal capillaries or tissue level. Pulmonary edema (abnormal accumulation of fluid in the lungs) can also result and causes laboured respiration, shortness of breath and coughing. Parasitised red blood cells can also adhere to microvesicles of the gastrointestinal tract resulting in symptoms of nausea, vomiting and loss of appetite. This has often lead to the misdiagnosis of viral gastroenteritis or hepatitis. One of the most commonly seen symptoms of *P. falciparum* malaria infection is anaemia. This can result from destruction of red blood cells from merozoite release or inhibition of hematopoiesis (production of red blood cells in the bone marrow) by TNF. Unlike the other three *Plasmodiums*, *P. falciparum* infects red blood cells of all ages, which allows infection of all circulating red blood cells (Virtual Naval Hospital, 1998).

1.5 Control of malaria

1.5.1 Chemotherapeutic targets and antimalarial drug discovery

The basic elements of any malaria control program require early diagnosis and correct treatment. The emergence of resistance to widely used antimalarial drugs, such as chloroquine, has elevated the need to discover and validate new targets to generate new drug candidates (Hastings and D'Alessandro, 2000). However, even despite the large collective effort of scientists around the world, the number of compounds tested against malaria remains very small. Of these compounds tested very few prove active in inhibiting parasite growth (Olliaro and Yuthavong, 1999).

The characteristics required of a putative drug target limit the field of targets available for study. The putative target needs to be an essential feature of the parasites life cycle and must differ significantly from any analogous process in the host. There must be no alternative pathways that could circumvent the target and the target must be accessible to the target-specific drug. The whole exercise of putative target evaluation and drug design is long, risky and expensive (Olliaro and Yuthavong, 1999).

Most targets that have been selected for antimalarial chemotherapy are in the parasites blood stages. This is only because blood-stage parasites can be cultured and tested with drugs, and is not by some planned strategy (Olliaro and Yuthavong, 1999). Validated chemotherapeutic targets have been summarised in Table 1.1.

Table 1.1: Summary of principal chemotherapeutic targets in *Plasmodium* (Olliaro and Yuthavong, 1999).

Pathway	Enzyme/process
Haemoglobin metabolism	Plasmepsin I, II (aspartic proteinases; cathepsin D-like) Falcipain (cysteine proteinase; cathepsin L-like) Haem polymerization, depolymerization Haem synthesis
Folate metabolism	Dihydrofolate reductase-thymidylate synthase (DHFR-TS) Dihydropteroate synthase-2-amino-4-hydroxy-6-hydroxy methyl-dihydropteridine pyrophosphokinase (PPPK-DHPS)
Pyrimidine synthesis, electron transport	Carbamoyl-phosphate synthase II Dihydroorotate dehydrogenase (DHODase) Cytochrome oxidase
Purine salvage	Hypoxanthine-guanine phosphoribosyltransferase
RNA/DNA	RNA polymerase Ribonucleotide reductase Topoisomerase I and II
Glucose transport, Glycolysis	**PfHT1** Lactate dehydrogenase (LDH)
Phospholipid metabolism	Phosphatidylcholine/phosphatidylserine synthesis
Artemisinin receptors	Iron haem as activator of protein alkylation
Oxidant damage	Potentiation of oxidant drugs
Oxidant defense	Glutathione reductase Superoxide dismutase (SOD)

These chemotherapeutic targets include processes occurring in the parasites digestive vacuole such as haemoglobin digestion, haem detoxification and antioxidant defence mechanisms. Other targets include enzymes involved in nucleic acid metabolism, phospholipid metabolism, glycolysis and tubulin assembly. Trafficking and signalling processes can also be targeted for antimalarial drugs. Such systems can be used to facilitate selective entry of antimalarial drugs, or to prevent key nutrients from being internalised (Olliaro and Yuthavong, 1999). A good candidate for this type of

antimalarial drug design, but still requiring further elucidation, would be the *P. falciparum* hexose transporter (PfHT1), isolated and sequenced by Woodrow *et al* (Highlighted in bold in Table 1.1; Woodrow *et al*, 1999).

A proposed strategy in treating malaria is to use antimalaria drugs in combination (Hastings and Alessandro, 2000). These combinations give old drugs new uses, which is beneficial for the limited amount of drugs in use (Winstanley, 2000). Drugs used for the treatment of *P. falciparum* malaria are listed below.

Chloroquine is the main drug used against the fight of malaria. It is safe and cheap and is used extensively for treating outpatients (not hospitalised). However, resistance is now extensive, yet it remains the most commonly used first-line drug despite failure rates of over 70% in some areas in Africa (Winstanley, 2000).

Quinine is a naturally occurring compound with relatively low potency. Malaria parasites satisfy most of their amino acid needs by consuming haemoglobin. Haem is the by-product of this digestion and is detoxified through polymerisation. Quinine is thought to act by binding to haem, preventing its polymerisation that would otherwise have reduced its toxicity to the parasite (Olliaro and Yuthavong, 1999). Resistance to the drug has developed, yet clinical failure is low outside Southeast Asia. Intravenous infusion is the preferred administered route, but it can be taken orally if it is taken at least twice daily. This makes it a difficult drug to use in outpatients (Winstanley, 2000).

Artemisinin and its derivatives were first discovered in an herbal remedy, and since then semisynthetic derivatives have been developed. This allows for greater antimalarial potency, but they are more expensive than the naturally occurring compound (Winstanley, 2000). It was hoped that these drugs would help reduce the mortality of severe malaria, but trials have shown that the drug has no clinical advantage over quinine. However it can be used where quinine resistance is evident (Van Hensbroek, 1996).

Pyrimethamine is more commonly used in combinations with other drugs. Sulfadoxine-pyrimethamine (SP) is cheap and is the most widely used combination (Winstanley, 2000). It eliminates slowly and therefore only one dose is needed. SP is however prone to rapid emergence of resistance and is used only for uncomplicated cases of *P. falciparum* malaria because of its slow effect (Winstanley, 2000).

Mefloquine is used for uncomplicated malaria. It is expensive and therefore is not in general use throughout Africa. Resistance has been noted in parts of Southeast Asia (Winstanley, 2000).

Halofantrine is an expensive drug and has been associated with ventricular arrhythmias (disturbances in ventricle rhythm), which has led to its reappraisal. However with resistance emerging to other available drugs, this drug remains in use as an alternative (Winstanley, 2000).

1.5.2 Vaccine development

Pre-erythrocytic vaccines; sporozoites and the intrahepatocytis stages. Rational design of malarial vaccines, like any other vaccine, requires characterisation of the protective immune response and identification of the antigens that elicit the response. Vaccines developed with the help of this information will elicit an immune response identical to that of the parasite antigen (Heal *et al*, 2001). During the past decade major efforts have been made to develop vaccines against the pre-erythrocytic malaria stages. Pre-erythrocytic vaccines may prevent sporozoites entering hepatocytes, or may inhibit parasite development within the hepatocyte (Kwiatkowski and Marsh, 1997). When the malaria parasite invades hepatocytes in the liver, these hepatocytes express antigens via the endogenous pathway and present it to $CD8^+$ T cells in association with major histocompatibility complex (MHC) class I molecules. However most vaccines deliver exogenous antigens via the MHC class II pathway to the host's immune system, inducing a $CD4^+$ T cell response (Heal *et al*, 2001). Evidence also suggests the possible involvement of $CD4^+$ T cells in sporozoite immunity (Tsuji *et al*, 1990). The challenge for the future is to develop a pre-erythrocytic vaccine capable of inducing protective antibodies against sporozoites, as well as inducing $CD8^+$ and $CD4^+$ T cell responses against the hepatocyte stage (Oliveira-Ferreira and Daniel-Ribeiro, 2001).

Vaccines target sporozoites and the intrahepatocyte stages mentioned above, as well as selective antigens of asexual blood stages. There is also the transmission blocking vaccines against gametocytes, gametes, or later stages in mosquitoes (Abath *et al*, 1998). Since one sporozoite is capable of developing into severe blood stage

infection, antisporozoite vaccines must be 100% effective. Vaccines developed from irradiated sporozoites resulted in complete resistance to sporozoite challenge in immunized mice (Jones and Hoffman, 1994).

Merozoite target antigens for vaccine development. Plasmodium species belong to the phylum Apicomplexa, so called for their apical secretory organelles named rhoptries and micronemes. During erythrocyte invasion merozoites transfer rhoptry proteins from these organelles to the erythrocyte surface to facilitate entry into the erythrocyte (Holder *et al*, 1994). Merozoites have been found to possess a number of surface antigens that could assist in erythrocyte adhesion and immune evasion. These surface proteins have been the focus of vaccine development since they represent a group of antigens that the host primarily is exposed to (Howard and Pasloske, 1993). These include the 195 kDa merozoite surface antigen 1 (MSA-1), which is thought to facilitate host immunity evasion and erythrocyte adhesion (Howard and Pasloske, 1993), and merozoite surface antigen-2 (MSA-2; 45kDa). Monkeys immunised against *P. falciparum* MSA-1 were protected against asexual parasite challenge (Etlinger *et al*, 1991), and mice immunised with *P. falciparum* MSA-2 produced antibodies against MSA-2 and protected the mice from challenge by murine malaria *P. chabaudi* (Saul *et al*, 1992).

Other merozoite proteins targeted for vaccine development, which are not found on the merozoites surface, include the apical merozoite antigen (AMA-1, 83kDa), which was first isolated from *P. knowlesi*. AMA-1 induces antibodies in monkeys that protect against further merozoites invasion (Deans *et al*, 1984), and homologues to AMA-1 have since been identified in *P. falciparum* (Preiser *et al*, 2000). Others include RAP-1/2 (80/42kDa) located in the rhoptries, ring-infected erythrocyte surface antigen (RESA, 155kDa) located in the dense granules, EBA-175 (175kDa) in the micronemes or apical end and SPf66, which is a synthetically constructed peptide polymer containing sequences from three merozoite proteins including MSA-1 (Howard and Pasloske, 1993; Hommel, 1990).

Infected erythrocyte membrane surface target antigens. Infected red blood cells express parasite produced antigens on the cell surface that elicits specific

IgG antibodies. PfEMP-1 (250-400 kDa) is one of these antigens and it accounts for sequestration of the IRBC to host endothelial cells in the blood vessels. This is the main cause of blood clots resulting in cerebral malaria that is experienced by patients infected with *P. falciparum* if left untreated. Sequestration of IRBC prevents circulation of these cells through the spleen where cells containing inclusions are systematically destroyed. Vaccine-elicited antibodies against PfEMP-1 may block IRBC sequestration and reduces the pathology of cerebral malaria, and will also allow for the IRBC to be destroyed by the spleen (Howard and Pasloske, 1993). Another similar protein is rosettin (22-28kDa) that mediates rosetting whereby IRBC adhere to each other at the rosettin sites and cause blockage of blood vessels. This gene has not yet been cloned, but like antibodies against PfEMP-1, antibodies against rosettin may reduce pathology of cerebral malaria. Other surface antigens include *P. falciparum* histidine rich protein-2 (PfHRP-2; 65-75kDa) and the high molecular weight protein Ag332 (2500kDa; Howard and Pasloske, 1993).

Infected erythrocyte soluble target antigens. The protein PfHRP-2 is not only expressed on the IRBC surface, but is also secreted into the surrounding plasma from the IRBC. This protein has been considered as a possible target for malarial vaccines, along with others including the serine rich protein (SERP), Ag2 and Ag7. The latter three proteins are released from the IRBC during rupture and release of merozoites. These proteins are released in large doses in relatively short periods of time and elicit host responses such as pro-inflammatory cytokine release mentioned in paragraph 1.4. It has been proposed that removal of these proteins by vaccine-induced antibodies specific for these compounds would stop the cascade of undesirable host responses (Howard and Pasloske, 1993).

1.5.3 Vector control

Female species of the *Anopheles* mosquito are the carriers of the malaria parasite and transmit the disease to humans during feeding. The vector biting behaviour and its longevity as well as its susceptibility to the *Plasmodium* parasite are all important for understanding the development of the parasite in the mosquito, and its transmission to

humans. The vectors biting and resting behaviour is an important factor in determining the use of insecticides (Toure', 1999).

The tools that exist for malaria vector control include chemicals such as DDT and other insecticides. Environmental management techniques such as eliminating standing water to reduce vector breeding; implementing the use of larvivorous fish and other mosquito larva eating predators as well as applying bacterial pathogens to malaria breeding sites can help implement vector control. Humans in endemic areas or experiencing epidemics of malaria can implement control by using repellents and bed nets, or simply by swatting vectors (Mwenesi, 1999).

Bed nets have been used for approximately 2000 years for personal protection against malaria (Lindsay and Gibson, 1988). The concept of treating bed nets with insecticides was first implemented in the Second World War by soldiers stationed in malaria areas (Harper *et al*, 1947). Insecticides used for the treatment of bed nets include DDT (Mwenesi, 1999); alpha-cypermethrin; cyfluthrin, deltamethrin; etofenprox and permethrin (Lines and Zaim, 2000).

1.5.4 Resurgence of malaria

Malaria is currently responsible for 2-4 million deaths annually resulting from the 300-500 million people who contract the disease, 10 000-30 000 of these cases are as a result of people travelling to endemic areas (Doolan and Hoffman, 2001). Spraying with DDT insecticide in the 1960s and 1970s was very successful in reducing the prevalence of malaria. However, because of the bad publicity created against the use of DDT and the high cost involved in using alternatives, resurgence occurred (Curtis and Lines, 2000). A severe case of resurgence of malaria was reported in Madagascar after the halt of DDT use in the 1960s, which resulted in a recovery of the vector causing an epidemic in 1988-1991 that killed thousands (Curtis and Lines, 2000).

Other causes of resurgence originate from the increase in antimalarial drug resistance. The emergence of drug resistant parasites to the drugs that were useful in the past means that new drugs are developed which often have side effects such as fatal heart

rhythms, fatal skin disease, neurological disturbances or gastrointestinal distress (Doolan and Hoffman, 2001). There is an overall lack in chemical diversity in the antimalarial drugs in use, which has led to cross-resistance between drugs (Olliaro and Yuthavong, 1999). By understanding the parasites metabolic processes, and its pathology, more rational drug and vaccine targets may be revealed and researchers may be able to provide solutions to this growing epidemic.

1.6 Parasite nutrient requirements and uptake

During its asexual cycle the malaria parasite invades the erythrocyte, a cell devoid of all intracellular organelles and incapable of *de novo* protein synthesis (Liem *et al*, 1994). Erythrocytes are specialists in carrying molecular oxygen (O_2) from the lungs to the tissues of the body and for carrying carbon dioxide (CO_2) in the opposite direction. Haemoglobin, which is responsible for the red colour of blood, is the oxygen-carrying protein in erythrocytes. The mature mammalian erythrocyte is further adapted by lacking a nucleus. The amount of oxygen required by the cell for its own metabolism is thus very low, and most oxygen carried can be freed into the tissues. Although they use glucose to produce energy necessary for their survival, they cannot synthesize protein; therefore reparative processes are not possible (Lodish *et al*, 1995). Hence the interior of the host erythrocyte represents a highly unusual extracellular environment for the parasite inside (Kirk, 2001).

The interior of the erythrocyte has high concentrations of K^+ and proteins, low levels of Na^+ and only trace levels of Ca^+. Therefore the invading parasite must provide its own means of regulating its chemical composition and obtaining nutrients from the host erythrocyte cytosol. The parasite must also contend with the hosts own metabolism and also take measures to avoid the hosts immune system and systematic destruction of red blood cells via the spleen (Kirk, 2001). Such measures require alterations of the host erythrocyte membrane such as knobs for sequestration discussed previously, and permeation pathways to allow the entry of nutrients, which will be discussed below.

1.6.1 Proposed parasite solute transport pathways

For solutes to gain entry into the intracellular parasite they first have to gain entry into the erythrocyte cytosol across the EM. From there the solutes move into the parasite by crossing the parasite vacuole membrane and the parasite plasma membrane (PPM; Foley and Tilley, 1998). Three possible models have been proposed for this molecular trafficking, namely the conventional model and two direct access models: the 'metabolic window' and the 'duct' (Kirk, 2001).

1.6.1.1 The conventional model

For the conventional model, solutes enter and leave the cell via the erythrocyte cytoplasm and have to cross the three separate membranes in passing from the blood plasma to the parasite cytoplasm. Solutes pass through the erythrocyte membrane via two different means (Kirk, 2001).

Firstly, a number of endogenous host cell transporters have been shown to have an increased activity in parasite-infected erythrocytes. Such transporters include the Na^+-K^+ pump, which has a two-fold increased activity compared to uninfected cells (Kirk *et al*, 1991). Another example is putrescine uptake in *P. knowlesi* infected cells. There is an increase in putrescine uptake in *P. knowlesi* infected monkey red blood cells, with a similar K_m to that of uninfected erythrocytes but a three-fold higher V_{max} (Singh *et al*, 1997).

Secondly, solutes gain access to the erythrocyte cytoplasm through new permeation pathways (NPP). These pathways are induced by the parasite and have very different properties to endogenous transporters and bestow on the host cell an increased permeability to a wide range of solutes (Kirk, 2001). These pathways are induced in the erythrocyte membrane 10-20 hours post-invasion. They have a broad specificity and are permeable to both organic and inorganic cations and anions, zwitterions and non-electrolytes (Kirk, 2001). NPP make a small contribution to the net uptake of glucose (Krishna *et al*, 2000), but have a low to negligible permeability to sucrose (M_r = 342; Ginsburg *et al*, 1985). It is therefore unclear whether NPP have a fixed size cut

off but they have been shown to accommodate compounds with a molecular weight as large as 613 dalton (Kirk, 2001).

Once in the erythrocyte cytoplasm the solutes diffuse to the parasite vacuole membrane and cross it via a nutrient channel. Once in the space between the parasite vacuole membrane and the parasite plasma membrane they are taken up into the parasite by either endocytosis or by transport pathways of varying specificity (Gero and Kirk, 1994; Desai *et al*, 1993).

1.6.1.2 The direct access model

In the direct access model, solutes gain access to the parasite cytoplasm by more direct means. Firstly, solutes may enter and leave the erythrocyte cytosol in the same ways as in the conventional model. From the cytosol, the solutes may gain access to the parasite by either endocytosis or by diffusing through channels that span both the parasite vacuole membrane and the parasite plasma membrane thereby connecting the two and creating a direct passage. The 'metabolic window' involves all three membranes in close opposition allowing substrate to be taken up in one step possibly through a channel connecting all three membranes. An alternative suggestion is that solutes may reach the parasite plasma membrane directly through a 'duct' and then be taken up into the parasite by endocytosis or by a nutrient channel in the parasite plasma membrane (Kirk, 2001).

1.6.2 Nutrient requirements

1.6.2.1 Glucose

The asexual stage malaria parasite and the erythrocyte it invades have no carbohydrate reserves and are completely dependent on monosaccharides supplied from the host's blood plasma. The rate of glucose consumption by *P. falciparum* is up to two orders of magnitude higher than that in normal uninfected-erythrocytes (Krishna *et al*, 2000). Glucose is the key carbon source for both the host erythrocyte and the parasite, and both depend on glycolysis for ATP production (Tanabe, 1990).

The earliest *in vitro* studies demonstrated that externally added glucose disappeared more rapidly from the medium surrounding infected erythrocytes than it did from the medium surrounding uninfected erythrocytes (Sherman, 1988). Non-metabolised analogues of D-glucose such as 2-deoxy-D-glucose, 3-*O*-methyl-D-glucose and 6-deoxy-D-glucose (2-DOG; 3-OMG and 6-DOG, respectively) can be used to study glucose transport thereby distinguishing transport from catabolic processes (Tanabe, 1990). The glucose analogue 3-OMG was used by Sherman and Tanigoshi (1974) to study the transport of glucose in *P. lophurae* (bird) -infected erythrocytes. They determined that the increased rate of entry of 3-OMG was related to parasite growth and they hypothesised that entry was mediated by simple diffusion as well as facilitated diffusion. This facilitated diffusion was hypothesised to be from the endogenous glucose transporter since the V_{max} increased 8-fold whilst K_m was the same as the endogenous transporter. Other studies involving mice parasite-infected erythrocytes revealed that whilst the uninfected erythrocyte was impermeable to L-glucose, permeability to L-glucose increased in parasite-infected erythrocytes, indicating transport via an alternative route and not via the endogenous glucose transporter GLUT1 (Tripatara and Yuthavong, 1986).

With the successful cultivation of *P. falciparum in vitro* (Trager and Jensen, 1976), permeability studies could be extended to human parasite-infected erythrocytes. Studies by Ginsburg *et al* (1986) using solute-induced haemolysis of *P. falciparum*-infected erythrocytes revealed that permeability to carbohydrates was selective. Based on the entry characteristics of a variety of substrates they postulated the involvement of a positively charged pore. Transport was later postulated to be via simple diffusion (Ginsburg and Stein, 1987), which would account for the stereo-specific indiscrimination observed in infected erythrocytes with L- and D-glucose.

Tanabe (1990) postulated that L- and D-glucose gained entry into the infected erythrocyte cytoplasm by parasite induced NPP, and then crossed the parasite plasma membrane via an H^+:D-glucose cotransporter. An ATPase coupled proton pump would then replenish the protons, generating an electrochemical proton gradient across the parasite plasma membrane (Tanabe, 1990). Kirk *et al* (1996) set out to determine whether transport across the IRBC membrane was via an active transport

process or rather a passive (equilibrative) transport process. By using non-metabolised glucose analogues 2-DOG and 3-OMG they noted that the analogues failed to accumulate in the infected erythrocytes to levels above those of the external medium. They therefore determined that transport of glucose into the infected erythrocyte was via a passive glucose transport system, and not an active one (Kirk *et al*, 1996). This was later confirmed with the glucose analogue 6-DOG (Goodyer *et al*, 1997). By using cytochalasin B, a potent inhibitor of GLUT1, Goodyer *et al* postulated that most of the glucose transported into parasite-infected cells was via this endogenous transporter, and that a small amount was transported via NPP. This additional transport via NPP would account for the small levels of L-glucose transported into the parasite-infected erythrocyte (Goodyer *et al*, 1997).

A *P. falciparum* hexose transporter was recently isolated and sequenced by Woodrow *et al* (1999). By using an immunolocalization technique with antibodies raised against the N-terminal (residues 6-21) of PfHT1 and immunofluorescence they determined the transporter to be on the PPM. The stage specific expression of PfHT1 mRNA was monitored in synchronous *P. falciparum* cultures relative to that of a housekeeping gene β-tubulin. An early peak of mRNA expression was detected at the ring stage, which represents a time point of parasite development 8-16 hours after invasion. After 16 hours PfHT1 mRNA levels drop and increase again to intermediate levels during trophozoite and merozoites development (Woodrow *et al*, 1999).

Unlike the mammalian glucose transporter GLUT1, PfHT1 is capable of transporting fructose as well as glucose. Human erythrocytes also have a separate fructose transporter GLUT5 (Concha *et al*, 1997). It is therefore postulated that monosaccharides, glucose and fructose gain entry into the parasite by first crossing the IRBC via GLUT1 and GLUT5. Small amounts of monnosaccharides also cross the IRBC via NPP, accounting for stereo-isoforms (L-glucose) not usually permitted into uninfected erythrocytes. Substrates then cross the parasite vacuole membrane via high-capacity, non-selective channels. Both glucose and fructose are then transported across the parasite plasma membrane by PfHT1 (Figure 1.2; Krishna *et al*, 2000).

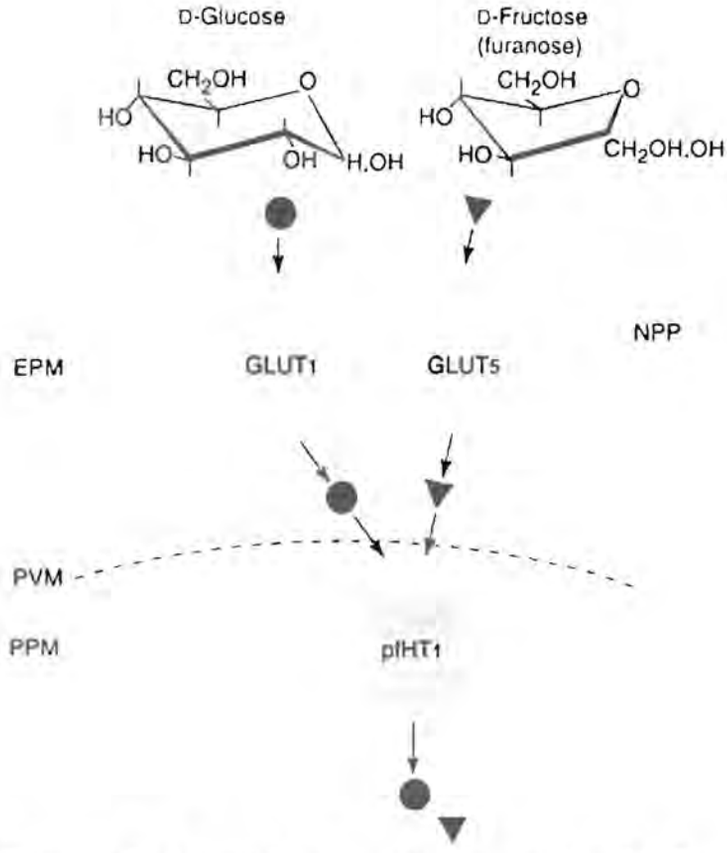

Figure 1.2: A model for hexose transport in the *Plasmodium falciparum* infected erythrocyte (Krishna *et al*, 2000). Glucose is represented by circles and fructose is represented by triangles. EPM, erythrocyte plasma membrane; PPM, Parasite plasma membrane; PVM, Parasite vacuole membrane; EPM, Erythrocyte plasma membrane; GLUT1, Mammalian glucose transporter 1; GLUT5, Mammalian glucose transporter 5; pfHT1, *P. falciparum* hexose transporter 1; NPP, New permeation pathway.

Since it is postulated that the majority of glucose and fructose gain entry into the IRBC by being transported via the endogenous GLUT1 and GLUT5 transporters, it is relevant to consider the transport properties of the uninfected erythrocyte membrane. The most active transporter in human erythrocytes is GLUT1, which comprises 3-5% of the total erythrocyte membrane proteins (Gould and Holman, 1993). GLUT1 was first isolated in the early 1980s by Baldwin *et al* (1982). To date 5 plasma membrane facilitated sugar transporters have been isolated and cloned (GLUT1-5; Walmsley *et al*, 1998) and a further 3 have been identified, namely the pseudogene GLUT6 (Kayano *et al*, 1990), the microsomal glucose transporter GLUT7 (Waddell *et al*, 1992) and the blastocyst glucose transporter GLUT8 (Carayannopoulos *et al*, 2000). These transporters fall into the major facilitator superfamily (MFS) also called the uniporter-symporter-antiporter family. Most of these families consist of proteins

predicted to have 12 transmembrane segments. The remainder have 14 predicted transmembrane segments (Pao *et al*, 1998).

The 12-transmembrane structure was first predicted for GLUT1 by Mueckler *et al* (1985) from analysis of a hydropathy plot, which was predicted from the transporter amino acid sequence (Figure 1.3). One *N*-linked oligosaccharide was predicted to be localised to either the C- or N-terminal third of the molecule at Asn^{45} or Asn^{411}. From the hydropathy plot Asn^{411} was predicted to lie within the membrane-spanning domain, therefore they predicted Asn^{45} to be the glycosylated site (Mueckler *et al*, 1985). By using *N*-glycasane treated human glucose transporter reconstituted in liposomes, this *N*-linked oligosaccharide was determined to be essential for glucose transport activity (Feugeas *et al*, 1990). From the hydropathy plot GLUT1 was predicted to have a small N-terminal hydrophilic domain assigned to the cytoplasmic face, and a larger hydrophilic C-terminal also on the cytoplasmic side (Mueckler *et al*, 1985). These findings were confirmed by proteolytic digestion with trypsin (Cairns *et al*, 1987).

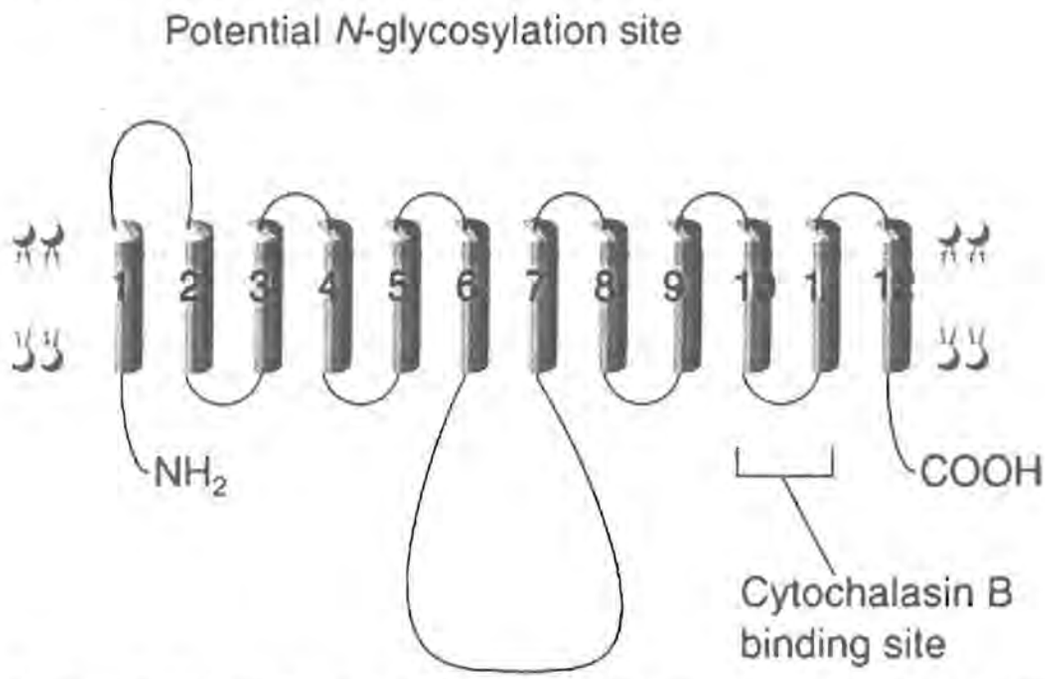

Figure 1.3: Schematic model of GLUT1 based on Mueckler's original proposal (Mueckler *et al*, 1985). Both the N- and C-terminal ends face inward. The *N*-glycosylation site lies between helices 1 and 2. A large endofacial loop lies between helices 6 and 7.

The transporter has both inward and outward facing sugar-binding sights predicted from binding of ethylidene glucose and cytochalasin B that fix the transporter in its

exofacial (outward facing) and endofacial (inward facing) conformations respectively (Walmsley, 1988). The kinetic mechanism of sugar transport is thought to be by sugar binding to the endofacial site causing a conformational change, which translocates the sugar across the membrane to be released from the exofacial conformation. Therefore sugar translocation occurs as the transporter oscillates between these conformations (Walmsley *et al*, 1998).

1.6.2.2 Amino acids and peptides

It was already established more than 40 years ago that the malaria-infected erythrocytes had an increased permeability towards amino acids (Sherman, 1988). As with uptake of exogenously supplied glucose, uptake of amino acids was dependent on the parasite species, size and its environmental condition (Sherman, 1988). The malaria parasite is thought to have limited ability to synthesise amino acids and most of its required amino acids are therefore derived from alternative sources (Kirk, 2001). The parasite is able to obtain amino acids in three ways: biosynthesis from other carbon sources, uptake from external media and catabolism of host haemoglobin (Kolakovich *et al*, 1997).

The parasite utilises haemoglobin as its primary source of amino acids. This hypothesis is supported by a 25-75% decrease in haemoglobin content of infected erythrocytes (Rosenthal and Meshnick, 1996). An acidic organelle called the digestive organelle is the site of haemoglobin proteolysis. The ingestion of the haemoglobin occurs through a structure called a cytosome. This is a mouth-like apparatus, which opens through the double membrane separating the parasite and erythrocyte cytoplasms. The cytosome buds off in the parasite forming a transport vesicle that then joins with the digestive vacuole where the haemoglobin is degraded (Rosenthal and Meshnick, 1996).

Inside the digestive vacuole two aspartic proteases, plasmepsins I and II, and one cysteine protease, falcipain, cleave haemoglobin into peptides. It was previously thought that peptides were degraded into single amino acids inside the digestive vacuole, but recent work by Goldberg's group contradicts this idea (Kolakovich *et al*,

1997). According to this work peptides are transported out of the vacuole and into the cytoplasm where exopeptidase convert the peptides into individual amino acids.

The short-term survival of *P. falciparum in vitro* requires exogenously supplied isoleucine and methionine, and growth over longer periods requires the supply of glutamine, glutamate, cysteine, proline and tyrosine to the growth medium (Divo *et al*, 1985). However, even amino acids that are not essential to the parasite are taken up from the extracellular medium (Kirk, 2001). The erythrocyte plasma membrane has a variety of different amino acid transport systems, and the majority of required amino acids seem to be transported via these endogenous transporters (Kirk, 2001). Glutamate does not gain entry via the endogenous transporters (Kirk, 2001) and may gain entry into the erythrocyte via NPP (Ginsburg *et al*, 1985). Whether amino acids gain entry into the infected erythrocyte via endogenous transporters or parasite-induced pathways still remains to be elucidated.

1.6.2.3 Nucleosides

Unlike mammalian cells, the malaria parasite is incapable of *de novo* synthesis of the purine ring. The parasite is therefore dependent on transporters and salvage pathways for purine nucleotide synthesis from host precursors (Parker *et al*, 2000). A number of purine salvage enzymes have been identified in the parasite, for example hypoxanthine-guanine-xanthine phosphoribosyltransferase (Keough *et al*, 1999). The parasite is however able to synthesise pyrimidines, and salvage pathways have not been identified for pyrimidine rings. Several antimalarial drugs target *de novo* pyrimidine metabolism, for example sulfadoxine is an inhibitor of parasite dihydropterate synthase and pyrimethamine and cycloguanil inhibit dihydrofolate reductase (Parker *et al*, 2000).

The host erythrocyte has endogenous nucleoside transporters, which are enantiomerically selective (Upston and Gero, 1995). The endogenous nucleoside transporters in infected erythrocytes are responsible for 50% of the total nucleoside transport into the cell and are potently inhibited by a structural analogue of adenosine 6-((4-nitrobenzyl)thio)-9-β-D-ribofuranosylpurine (NBMPR). The parasite derived nucleoside transporters are distinct from the endogenous transporters in their inability

to be inhibited by NBMPR and were found to be a combination of nonsaturable and carrier-mediated transporters (Upston and Gero, 1995).

Recently the *Xenopus* oocyte expression system has been used to express and characterise a *P. falciparum* encoded nucleoside transporter. This transporter was identified by two independent research groups and was designated two different names, PfENT1 (Parker *et al*, 2000) and PfNT1 (Carter *et al*, 2000). The nucleoside transporter in each paper has an identical protein sequence, but there are discrepancies regarding its substrate affinities between the two studies. In the study by Carter *et al* the K_m for D-adenosine was reported to be 13,2 μM, and in the study by Parker *et al* the K_m was determined to be 320 μM. Other discrepancies included the transport of nucleobases such as hypoxanthine. Where PfNT transport of D-adenosine was not inhibited to any significant degree by hypoxanthine, PfENT1 was with a K_m for hypoxanthine of 0.41 mM. It was also reported that PfNT1 was 85% inhibited by dipyridamole, whereas PfENT1 was not inhibited at all by dipyridamole (Carter *et al*, 2000; Parker *et al*, 2000). Further elucidation of this transporter is required.

1.7 Trafficking and secretion in *Plasmodium*

Understanding the malaria parasites trafficking mechanisms can give scientists a better understanding of this pathogen, as well as help to identify potential antimalarial targets. Since *P. falciparum* is a eukaryotic organism, it can be assumed that the organism will have similar trafficking mechanisms to other eukaryotes for the synthesis and export of proteins.

1.7.1 General eukaryotic secretion

Secretory activities are conserved in higher eukaryotes that have been studied to date, which include yeasts and mammals. The largest membrane in an eukaryotic cell is the endoplasmic reticulum (ER). The smooth ER is the site of synthesis and metabolism of fatty acids and phospholipids. The rough ER synthesizes certain membrane and organelle proteins as well as all proteins to be secreted from the cell. A signal sequence on all newly synthesized secretory proteins targets them to the ER. This sequence is 16-30 amino acids long and directs the ribosome to the ER membrane and

initiates the transport of the growing polypeptide across the membrane. The chaperone Bip binds to the growing chain on the ER luminal surface and with the help of hydrolysis of ATP, releases the newly synthesized chain into the lumen of the ER. Bip also facilitates the eventual folding of many secretory proteins. Cytosolic proteins are released into the ER lumen or the ribosome is released from the ER membrane and continues to synthesise the membrane protein in the cytosol. Enzymes on the luminal side of the ER add carbohydrates to the chain (Lodish *et al*, 1995).

Several minutes after their synthesis, most proteins leave the rough ER within membrane-bounded transport vesicles that bud off from the ER. The vesicles carry the proteins to the Golgi complex, which has three defined sections: the *cis*, *medial* and the *trans*. The transfer vesicles fuse with the *cis* region of the Golgi where they deposit their proteins. The proteins then progress from the *cis* to the *medial* to the *trans* regions where different enzymes modify secretory and membrane proteins differently. After modification the proteins are transported out of the complex by a second set of transport vesicles that bud off from the *trans* domain. The transport vesicles fuse with the plasma membrane resulting in the release of its secretory proteins, or the fusion of its membrane bound protein with the plasma membrane (Lodish *et al*, 1995).

1.7.2 Trafficking of proteins within the malaria parasite

In order to get to the IRBC membrane cytosolic, surface and parasite derived proteins have to transverse the PPM, parasite vacuole membrane and through the IRBC cytosol (Foley and Tilley, 1998). Classical secretory mechanisms such as those mentioned in Paragraph 1.7.1 may be involved to take the protein as far as the PPM, but thereafter secretory mechanisms involved in taking the protein to its final destination are speculations.

1.7.2.1 The parasitic endoplasmic reticulum

Since the first application of the electron microscopy to malariology in 1957, evidence has existed for the presence of an ER that was observed in the parasitized

cell (Aikawa, 1971). Further evidence for a parasitic ER can be gained from proteins which transverse through the ER on their way to their final destination. These proteins can be detected by immunoelectron microscopy close to the cytoplasmic side of the parasite nuclear membrane where an ER would be located. One such protein is PFGCN20, which is located in the lumen of the parasite vacuole (PV) or in PV protrusions. PFGCN20 is an ATP-binding cassette protein and is closely related to the yeast translation regulator Gcn20p. It has been detected in membranous structures at the side of the nuclear membrane before transport to the PV (Bozdech *et al*, 1998).

Homologues of trafficking components such as the 78 kDa glucose-regulated protein (grp78) identical in sequence to the globulin-binding protein Bip, whose function is summarized in Paragraph 1.7.1, has also been identified (Kumar *et al*, 1988). This homologue in *P. falciparum* is called Pfgrp and has a carboxylic terminus Ser-Asp-Glu-Leu (SDEL; Kumar *et al*, 1991). This is similar to mammalian grp78 proteins that have a C-terminus, Lys-Asp-Glu-Leu (KDEL) and in yeast cells that have a C-terminus His-Asp-Glu-Leu (HDEL; Kumar and Zeng, 1992). This C-terminus sequence is essential for the proteins retention in the lumen of the ER (Munro and Pelham, 1987). Synthesis of grp78 is induced by the presence of misfolded protein and forms tight bonds with misfolded proteins and assists in their proper assembly and folding (Kassenbrock *et al*, 1988). Using a highly specific antiserum in an investigation by immuno-gold electron microscopy, Pfgrp was localized in the cytoplasm of the parasite in ER-like membranous structures (Kumar *et al*, 1991).

1.7.2.2 An alternative ER-like organelle

Studies have shown that many *Plasmodium* proteins that are destined for export are not processed by the ER and Golgi, but are exported via an alternative pathway (Wiser *et al*, 1997). This is based on blockage experiments with Brefeldin A (BFA). BFA is a unique fungal antibacterial agent that has a 13-membered macrocyclic lactone ring (Misumi *et al*, 1986). BFA is known to inhibit protein secretion in higher eukaryotes by disrupting the integrity of the Golgi apparatus (Benting *et al*, 1994). In a study conducted by Misumi *et al* (1986) they attempted to determine the effect of BFA on the transport of secreted proteins. They found that BFA had little effect on

protein synthesis but had a dose-dependent inhibitory effect on protein secretion from the classical secretory pathway. Treatment with BFA caused a marked alteration in the ER. The ER with or without ribosomes was markedly dilated and contained unstructured material in the ER lumen, which they postulated to be secretory proteins (Misumi *et al*, 1986).

Lippincott-Schwartz *et al* (1989) established that in BFA treated murine cells *cis/medial* Golgi compartments redistributed to and became part of the ER. This resulted in glycoproteins being processed by *cis/medial* Golgi enzymes within the ER lumen. The effect of BFA was reversed upon removal of the drug. Golgi proteins were then transported from the ER into a newly formed Golgi whilst ER-resident proteins, with which they had been mixed, remained in the ER (Lippincott-Schwartz *et al*, 1989).

Treatment of *Plasmodium* infected rodent cells with BFA caused the formation of a single BFA-induced compartment in which secreted proteins such as Pb(em)65, a *P. berghei* erythrocyte membrane located protein, accumulated (Wiser *et al*, 1997). After BFA treatment, Pb(em)65 was associated with the parasite in a single BFA-induced structure that was observed at the parasite periphery. A similar pattern was observed for Pb(ec)13 and Pb(ec)31, which are normally localized to non-membrane bound inclusions in the erythrocyte cytoplasm. A parasite protein that resides in the lumen of the ER, Pfgrp, was used as a control and was found to accumulate in membrane vesicles at the cytosolic side of the nucleus after treatment with BFA. This suggests that Pfgrp remains in the ER and is not affected by BFA treatment, and that the inclusion at the parasite periphery was not the ER. Therefore Pb(em)65 was not transversing through the ER but accumulated in a previously undetected compartment. This ER-like organelle located at the parasite periphery may specialize in export of proteins to the erythrocyte cytoplasm and EM. Wiser *et al* proposed to call this ER-like compartment sERA: secondary ER of apicomplexa (Wiser *et al*, 1997).

1.7.2.3 Golgi vesicles

The Golgi is the major site for sorting of proteins and lipids in eukaryotic cells. *N*-glycosylation is one of the major markers used for Golgi detection, since *N*-glycosylation occurs in the ER and the *N*-glycosylated protein can then be monitored as it transverses through the Golgi vesicles. PfHT1 is predicted to have *N*-linked oligosaccharides. Potential glycosylation sites (NXT or S, where X is any amino acid) are at asparagines 69 and 72 (Woodrow *et al*, 1999) located similarly to GLUT1 asparagine 45 (Meuckler *et al*, 1985). *N*-linked oligosaccharides in *P. falciparum* are predicted to occur at low concentrations and therefore have not been used as a Golgi marker (Gowda and Davidson, 1999).

An enzyme that is normally associated with the Golgi, sphingomyelin synthase, is one Golgi marker that has been detected in the malaria parasite (Haldar *et al*, 1991). Uninfected erythrocytes are incapable of sphingomyelin synthesis. Studies with both fluorescent and radiolabelled ceramides have shown that ceramides are converted to sphingomyelin in infected but not in uninfected erythrocytes. The levels of sphingomyelin in the host cell membrane are decreased upon infection with *P. falciparum*, which suggests that the host's sphingomyelin is internalised and degraded to ceramide by the parasite and presumably re-converted to sphingolipids in the Golgi apparatus (Haldar *et al*, 1991).

The binding and metabolism of a fluorescent ceramide analogue *N*-[7-(4-nitrobenzo-2-oxa-1,3-diazole)] amino-caproyl sphingosine (C6-NBD-cer) was investigated in *P. falciparum* infected cells. Earlier studies on live mammalian cells showed that C6-NBD-cer was metabolised to C6-NBD-cerebrosides and C6-NBD-sphingomyelin (C6-NBD-Sm), providing *trans* Golgi markers (Pagano *et al*, 1989). In *P. falciparum*-infected erythrocytes these markers were found to accumulate in the parasite vacuole membrane and flattened cisternae in the infected-erythrocyte cytosol (Haldar *et al*, 1991; Elmendorf and Haldar, 1994). This was the first report on the distribution of Golgi markers and also raised speculation on Golgi-like elements beyond the parasite membrane. This is comparative to other eukaryotic studies such as those done on rat liver cells where 87% of sphingomyelin synthase activity was found in the Golgi and

the remaining 13% in the plasma membrane, suggesting a second site of sphingomyelin activity in cells (Elmendorf and Haldar, 1994).

A *P. falciparum*-derived protein homologous to ERD2, a Golgi marker in higher eukaryotes, was discovered. For soluble proteins in the ER the signal for retention is a tetrapeptide sequence at the C-terminus mentioned in paragraph 1.7.2.1. ERD2 is found in the *cis*-Golgi and recognises this signal on polypeptides that stray from the ER and returns them. The *P. falciparum* ERD2 (PfERD2) has 42% identity to the higher eukaryotic ERD2. Immunofluoresent studies of PfERD2 conducted on parasitized cells localized it to a tightly defined perinuclear spot (Elmendorf and Haldar, 1993).

Additional Golgi markers have been identified that are members of a GTP-binding protein family namely rab1, rab6 and rab11. Like PfERD2, rab6 has been localised to tubulovesicular membranes in the parasite, with multiple dispersed sites in the ring stage and the trophozoite stage, and in closer proximity to the plasma membrane than that seen for PfERD2. This suggests that Golgi membranes lack cisternal structure and are rather tubulovesicular, and since they are also dispersed suggests that the Golgi elements in the parasite are not stacked as in mammalian Golgi (Van Wye *et al*, 1996).

1.7.3 Trafficking of proteins beyond the parasite periphery

As in any eukaryote, secreted proteins of *Plasmodium* can be transported within the parasite cell via conventional modes of transport such as the ER and Golgi apparatus, which will take them as far as the PPM. Some proteins need to transverse further than the parasite plasma membrane such as erythrocyte cytosolic parasite antigens and antigens presented on the infected erythrocyte membrane.

1.7.3.1 Proposed models of trafficking beyond the parasite plasma membrane and PVM

There is an added complexity to the protein trafficking in malaria infected erythrocyte in that the parasite needs to transport proteins beyond the confine of its own plasma membrane. To date two models have been proposed for the translocation of the secreted proteins across the parasite plasma membrane and parasite vacuole membrane (Lingelbach, 1997; Foley and Tilley, 1998).

1.7.3.1.1 The two-step model

In this model proteins may enter the ER and are secreted into the PV via a classical vesicle-mediated pathway (Figure 1.4, steps 1 and 2). All secreted proteins are targeted to the PV and are sorted there. Once in the PV, proteins destined for the IRBC cytosol and the IRBC membrane will need to be translocated across the parasite vacuole membrane possibly via membrane pores (Figure 1.4, step 3; Foley and Tilley, 1998).

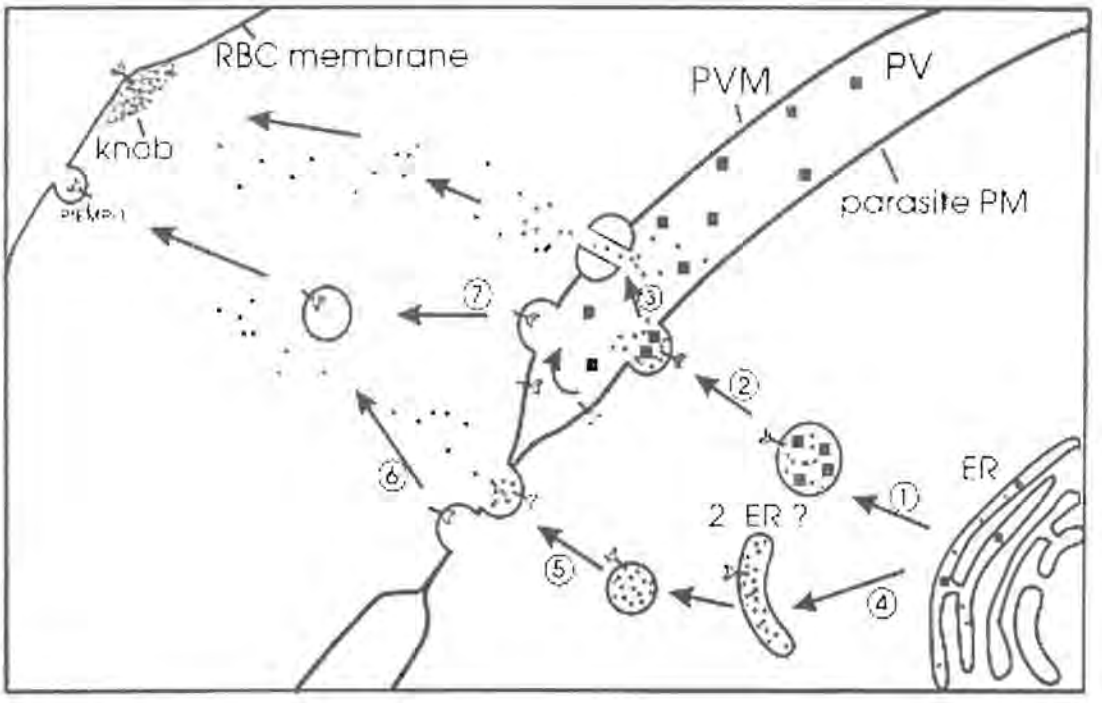

Figure 1.4: Models for sorting of proteins destined for different compartments in malaria-infected erythrocytes (Foley and Tilley, 1998). Proteins (squares) destined for the parasite vacuole (PV) remain trapped in the PV (Steps 1 and 2). Proteins (circles) that are destined for the erythrocyte cytoplasm or the red blood cell

membrane (RBC membrane) may be secreted into the PV and then translocated across the parasite vacuole membrane (PVM; Step 3). Alternatively, they may be sorted into a specialised secretory organelle (Step 4) and secreted directly into the erythrocyte cytoplams at a fused site in the parasite plasma membrane (parasite PM) and parasite vacuole membrane (Step 5). Transport to the red blood cell (RBC) membrane may occur by budding vesicles or by protein aggregate diffusion (Steps 6 and 7).

1.7.3.1.2 The single-step model

In this model malaria proteins located beyond the parasite plasma membrane are targeted to sites where the parasite plasma membrane and parasite vacuole membrane have fused. Therefore the proteins are translocated across two membranes in a single step. The proteins are contained in transport vesicles. Since some proteins would be transported to the PV or parasite vacuole membrane and some to the RBC cytosol and RBCM, two different types of transport vesicles would be required. One type of vesicle would target proteins to a PPM-PVM fusion site for transport to the IRBC membrane and IRBC cytosol (Figure 1.4, steps 4-5). The other type of vesicle would fuse to the parasite plasma membrane to release proteins into the PV (Figure 1.4, steps 1-2). This would require a detailed secretory mechanism apart from the ER, where the sERA possibly play a part (Figure 1.4, step 4; Lingelbach, 1997; Foley and Tilley, 1998).

1.7.3.2 Intra-erythrocytic membranes for transport beyond the PVM

The malaria parasite produces a complex membrane structure within red blood cells that it infects. The most prominent structure is the tubulovacuolar network (TVN), which is also known as the tubulovesicular membrane (TVM) originally coined by Elmendorf and Haldar (1993).

The tubovesicular membrane appears to be continuous with the parasite vacuole membrane and extends into the cytoplasm of the IRBC. Some researchers have claimed that the tubovesicular membrane functions to transport proteins to the IRBC membrane but have so far failed to find any evidence for a direct connection between the cytoplasmic side of the IRBC membrane and the extended arms of the tubovesicular membrane (Fujioka and Aikawa, 1993). The tubovesicular membrane

does however appear to have some function similar to the Golgi, since sphingomyelin synthase activity appears to be partially located within the tubovesicular membrane (Elmendorf and Haldar, 1994). However limitation of tubovesicular membrane growth by treatment with sphingomyelin synthase inhibitor does not prevent protein transport. The tubovesicular membrane also appears much later (33h post invasion) than parasite antigen presentation on the IRBC membrane. The necessity of the tubovesicular membrane for protein export is therefore debatable (Lauer *et al*, 1997).

1.8 Conclusion

The malaria parasite species most responsible for malaria related devastation, *P. falciparum*, is slowly building up resistance to the armoury of drugs used against it. Its vector, the *Anopheles* mosquito, is itself building up resistance to current insecticides and remaining options such as a vaccine remains elusive. The great hope for eradication of malaria held during the 1960s has not been realised and more people are exposed to malaria today than were during the last century.

Hope now exists in new drug target identifications that may lead to more affordable and effective drugs. To achieve this, researchers must focus on validated targets for drug design and identify targets by studying basic and metabolic processes essential for the parasites survival. One valid and obvious target would be to focus on the parasites main source of energy: glucose. The parasite has no glycogen reserves and is dependent on its host for its glucose supply. *P. falciparum* parasites are able to sustain life in fructose-substituted glucose-free medium and therefore if one parasite derived transporter exists for the transport of both monosaccharides to the parasite, this could be a valid drug target. Recently one such transporter was discovered: PfHT1 (Woodrow *et al*, 1999).

The research on the *P. falciparum* glucose transporter has only just begun, and compared to research on the mammalian glucose transporter GLUT1, much work is needed to elucidate the mechanism of this transporter to identify parasite specific properties. Already a discrepancy has emerged in that the PfHT1 transports both glucose and fructose, whereas GLUT1 does not. It has been hypothesised by Krishna

et al that glucose and fructose gain entry into the IRBC via GLUT1 and GLUT5, and a small amount via NPP (Krishna *et al*, 2000).

As yet no parasite derived hexose transporters have been identified except for PfHT1. Identification of the regions of PfHT1 responsible for glucose and fructose transport that distinguishes it from GLUT1 would be important information for drug design. Inhibiting PfHT1 is anticipated to have two advantages, that (1) diversion of essential glucose away from the parasite will prevent problems symptomatic of malaria such as hypoglycaemia, and (2) killing parasites may be more effective if inhibitors of PfHT1 are combined with common antimalarials than by using antimalarials alone (Woodrow *et al*, 1999).

1.9 Aims and strategy of the study

With the isolation, cloning and sequence analysis of the *P. falciparum* hexose transporter already accomplished, its characterisation is underway. So far little is known as to what distinguishes this transporter from all the other well described mammalian transporters (GLUT1-5). The main question is: what allows this transporter the ability to transport both glucose and fructose, whereas no such transporter exists for higher eukaryotes? This question has been addressed in a recent study conducted by Woodrow *et al* (2000) and is extended by research described in the current study.

Until a crystal structure becomes available of the facilitated glucose transporters, indirect methods have to be applied to elucidate their mechanisms. Indirect mechanisms methods include mutational studies applied to GLUT1 to identify helices within the transporter that are thought to arrange in an aqueous core and interact directly with the substrate. Glucose analogue studies can also aid the identification of carbon atoms on the glucose ring involved in protein-substrate interactions. Mutational studies also help identify amino acids essential for protein-substrate interactions to discover the properties needed for putative chemotherapeutic drugs to be affective inhibitors of glucose transport.

A mutational strategy based on PCR to produce point mutations will be outlined in Chapter 2. Kinetic studies on these point-mutated hexose transporters are described in Chapter 3 using a *Xenopus* oocyte expression system. Then finally studies with mammalian/ PfHT1 chimeras, along with Western blotting are described in Chapter 4. These results contribute towards a better understanding of the PfHT1 transporter of the malaria parasite.

t=0hrs, frozen samples from 3 Feb 02

	0	20	40	60	80	100	120	140	160	180
	0.23	0.213	0.208	0.197	0.184	0.17	0.156	0.139	0.119	0.104
	0.234	0.204	0.22	0.196	0.187	0.168	0.155	0.14	0.127	0.111
	0.228	0.207	0.21	0.199	0.186	0.173	0.156	0.144	0.119	0.111
AVE	######	0.208	0.2127	0.1973	0.1857	0.1703	0.1557	0.141	0.1217	0.1087
AVE-0.0	######	######	######	######	######	######	######	######	######	######
%ABS	100	87.792	90.305	82.047	75.763	67.504	59.605	51.706	41.293	34.291