CHAPTER 1: INTRODUCTION AND REVIEW OF LITERATURE
1 Introduction and Review of Literature

1.1 General Introduction to Malaria

Parasite borne diseases have been the scourge of humanity since the beginning of civilization. Awareness as to the nature of these organisms has increased over the course of time yet they still find ways to elude the varied and ever-evolving arsenal of anti-parasite pharmaceuticals. The most elusive of these diseases, as well as the most damaging to mankind is malaria. The word 'malaria' is from the vulgar Latin denoting the "bad (or evil) air" found around the Roman swamps where invariably people would come down with a debilitating, recurrent fever. The first clinical descriptions of malarial fever were given by Hippocrates in about 400 Before Christian Era (B.C.E.). By the 1600's, and possibly much earlier (Figure 1a), the bark of the cinchona tree was employed in Native American medicine for a malaria-like fever. The active component of the cinchona tree bark, quinine, was found to have an antimalarial effect. A century later Lancisi suspected the mosquito to be the carrier of malaria, but it wasn't until the nineteenth century that Alphonse Laveran won the Nobel prize for his discovery that malaria is caused by a protozoan in the blood (Lavaran, 1880). Sir Ronald Ross was awarded the Noble prize for his observation of the malaria parasite in mosquito that had fed on a human volunteer (Jarcho, 1984, and Hoffman, 1996). An Italian group elucidated the sporogony of \textit{P. falciparum} and \textit{P. vivax} in \textit{Anopheles} mosquitoes (Dobson, 1999). Surely the Victorian naturalist would not feel out of place as even today discussion about preventative measures considers bed-nets as the method of favour and microscopy performed by a trained eye as the best way towards diagnosis.

The World Health Organization (WHO) has stated that global eradication of malaria is impossible (WHO, 1987 and 2002). The current stance is to strive to keep the parasite and its resistance to drugs under control. Of the drugs currently in use, quinine has a century long history of use. Older synthetics such as pamaquine and mepacrine have given way to primaquine, mefloquine and artemisinin derivates. Even these are already developing pockets of resistance where compliance to correct drug regime is low (WHO, 1998). Thus the WHO is supporting the use of combinations of antiparasite drugs that work on different principles. The benefits of combinations like quinine and tetracycline, chlorproguanil and dapsone.
Figure 1a. Malaria Timeline

Indian medical texts describe fever with malaria-like symptoms. **1600 BCE**

Oldest known source of malaria DNA **450 CE**

Giovanni Maria Lancisi, a Roman doctor, notes that draining swamps curbs malaria. He suggests mosquitoes spread malaria but few listen. **1716**

British army doctor Ronald Ross observes malaria parasite in mosquitoes. **1897**

DDT developed **1937**

WHO launches global campaign to eradicate malaria. **1956**

WHO abandons malaria eradication in favour of control. **1967**

First *Plasmodium* gene cloned. Mutation leading to drug resistance found. **1983**

400 BCE Hippocrates describes clinical malaria and later (95BCE) Lucretius suggests that a microorganism might cause 'swamp fever'.

c.1640 Spanish colonists in South America discover quinine. The natives probably got to it first.

1880 French army surgeon Charles Louis Alfonse Laveran spots malaria parasite in blood.

1934 Chloroquine discovered in Germany. Forgotten and rediscovered in the 1940's.

1939-45 Second World War. The risk to soldiers prompts probably the most intense malaria research effort ever. Several new drugs discovered as a result.

1960's Drug-resistant parasites and DDT-resistant mosquitoes become widespread.

1979 Chinese researchers describe artemisinins, a new class of malaria drug.

2002 Gene sequences of *Plasmodium* and *Anopheles* completed

{after Sherman, 1998}

Figure 1b. Malaria Endemic Countries, 2000

{after WHO, 2000}
(LAPDAP), and sulfadoxime and pyrimethamine (SP) are that they are safe and more likely to be effective, as resistance to both drugs is less likely. Moreover, these are affordable drugs that are less likely to lack in compliance. Improper dosage, and inadequate and irregular reporting (WHO, 1996) are the primary man-made causes of resistance. SP and mefloquine are already going the way of chloroquine in developing resistance in most of South East Asia (WHO 2002). New drugs are not safe from misuse, as in one reported case where it was found that 38% of the artesunate purchased in South East Asia was fake (Newton et al., 2001), though it can be identified with a simple dye test (Green et al., 2000).

For effective control of the disease, mosquito control must work alongside antimalarial therapy. Fogging with pyrethrum, a natural insecticide that is harmless to mammals was superseded by less expensive, but highly toxic chlorinated chemicals such as 1,1,1-trichloro-2, 2-bis (p-chlorophenyl) ethane (better known as DDT), dieldrin and benzene-hexachloride (BHC). Many of these, such as organophosphates, like malathion, were originally developed in Nazi Germany as nerve gases for chemical warfare. Organophosphates have the advantage of being less stable than chlorinated compounds and thus easily broken down, and detoxified, naturally in the environment. A more modern derivative, parathion, is still being used today.

Control measures have targeted the aquatic habitat of mosquitoes since the beginning of the 20th century. An unlikely successful example is a decree by pre-world war two Italy's dictator that the malaria infested swamps in the Roman country-side must be completely dried out. This led to permanent eradication of the vector, and thus of malaria, from the entire country.

A concerted effort between malaria control and research is needed. Research is looking towards a better understanding of the mechanisms involved in the pathogenesis of malaria and for specific activity against one or another of the vital pathways of the *Plasmodium* life cycle in order to form a coordinated assault from different directions. Knowledge has expanded with regard to the underlying mechanisms of the pathology over the last decades, but many aspects of the molecular biology, immunology and epidemiology that govern the biologically complex pathogenesis as well as the renewed spread of this parasite are still unclear. The key is undoubtedly in the development and in the production of both new vaccines and new antimalarial drugs (Heddini et al., 2002).

1.1.1 The causative agent of malaria

The phylum Apicomplexa contains a large variety of organisms responsible for parasitic diseases in the tropical and sub-tropical regions of the world, the worst of which is malaria.
The causative agent of malaria is from the Protozoan family Plasmodiidae that contains only one genus: *Plasmodium*, and two subgenera: *Plasmodium* and *Laverania*. Only four *Plasmodium* species are responsible for malarial infection in man; *P. vivax*, *P. ovale*, *P. malariae* and *P. falciparum*. *P. vivax*, *P. ovale* and *P. malariae* belong to the *Plasmodium* subgenus. *P. falciparum* on the other hand belongs to the subgenus *Laverania* (Esposito et al., 1991) and is the only species that may lead to severe, often fatal, complications of the disease. In addition to these four human malaria parasites, man is occasionally exposed to infection with simian malaria parasites such as *P. cynomolgi* and *P. knowlesi* (Coatney et al., 1971).

The vector largely responsible for distribution of malaria is the female *Anopheles* mosquitoes (see the exhaustive work on the malaria vector, ‘Indian Anophelines’ by Nagpal and Sharma, 1995). The major foci occur in Africa, India, South-eastern Asia, and the Central and South Americas (Figure 1b). In all of its forms, the disease can be considered the most costly in terms of mortality and morbidity, sterile words that, in practical terms, stand to indicate the loss in human lives and the loss in the capacity of work to be done. *Plasmodium* is divided into many species; of the 121 species of *Plasmodia*, 22 infect primarily primates, 19 infect rodents, bats or other mammals, and 70 infect birds and reptiles (Bruce - Chwatt, 1985).

1.1.2 The burden of malaria

In figures it has been estimated that about 40% of the world’s population, some two billion people, are at risk from malaria infection and approximately 200 million are actually infected. In one year, an estimated 150 million people will develop the disease anew and over two million people will die (Heddini et al., 2002). The majority of deaths occur in children, of which 3000 under the age of five perish each day. With the number of deaths exceeding that from AIDS, malaria is easily the world’s most serious infectious disease, killing more people than any other communicable disease except tuberculosis (Persidis, 2000).

*P. vivax* infections account for more than half of the cases of malaria outside Africa and approximately 10% of the malaria in Africa. It is also prevalent in many regions of Asia and the Latin American subcontinent with niches where *P. vivax* is transmitted exclusively. *P. vivax* survives under more stringent conditions for transmission than *P. falciparum* and will thus prevail when, and if, *P. falciparum* transmission is finally controlled (Mendis et al., 2001). Though less deadly than *P. falciparum*, the characteristic relapses make *P. vivax* malaria a long lasting infection with devastating morbidity. The increased burden falls on endemic populations largely in the third world as well as on tourists. Even non-tourists are
susceptible to 'airport malaria' (Jafari et al., 2002), where infected mosquitoes carried aboard an airplane can infect individuals residing near airports, to bring home the importance of malaria research to countries that would otherwise know it solely from articles in seldom read journals. Moreover, data presented from many endemic regions showed that *P. vivax* infections are significantly under-diagnosed microscopically, and often samples are taken improperly and thus give rise to incorrect epidemiological information. It is here that the advent of new, rapid and accurate *vivax* diagnostic tools that can detect low parasitemia in mixed malaria infections would be useful to both research and control efforts (Mendis et al., 2001).

In fact, the disease known as malaria actually covers a wide variety of clinical outcomes that are dictated by factors tied to the nature of the parasite and to the host’s genetic, physical, behavioral and economic condition. Characteristic symptoms of malaria infection include paroxysms, splenomegaly, severe anemia and periodicity of the fever. Periodicity corresponds to the synchronized maturation of erythrocytic schizonts and their subsequent release of merozoites and toxic substances into the bloodstream (Miller et al., 1994). This causes chills, shivering, headache and a rise in body temperatures that can reach 45°C. The paroxysms continue for up to six hours and end with profuse sweating. They return after a number of days, thus giving physicians the original definition of the different types of malaria infection: tertian or quartan fever (*P. vivax* and *P. ovale* have a period of 48 hrs so the fever is tertian, in *P. malariae* it is 72 hrs resulting in quartan malaria, whereas *P. falciparum* is considered apart with its 48 hour or daily period). This periodicity also defines a prepatent period that is the minimal time elapsing between the initial sporozoite infection and the first appearance of the parasite in the erythrocytes, and the incubation period, defined as the time elapsing between infection and the first clinical manifestations. The prepatent period is characteristic of each species and is a good criterion for the diagnosis of the infecting parasite. For the human malarials they are: 5 days for *P. falciparum*, 8 days for *P. vivax*, 9 days for *P. ovale*, and 15 days for *P. malariae*. The incubation period of some species can be very long, *P. cynomolgi* can incubate for 102 days and *P. vivax* up to 275 days. A relapse occurs when the infection returns anew after an interval greater than the incubation period. Relapse is attributed to reactivation of hypnozoites, a hibernating form of *P. vivax*, *P. ovale* and *P. cynomolgi*, from hepatic parenchyma (Krotoski et al., 1980 and 1982a-d, and Newton and White, 1999).

Some forms of malaria are lethal to their host, such as *P. falciparum* in man and *P. knowlesi* in rhesus monkeys. *P. falciparum* can cause cerebral malaria, which is often fatal. Initial symptoms are neurological signs such as lethargy and stupor as well as acute changes in
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personality. Disturbances in movement can peak with seizures that eventually lead to coma. Parenteral chloroquine is given in case the patient is unconscious. In case of chloroquine resistance, an injectable form of artemisinin has seen success as a life saving drug in terminal patients (WHO, 1997 and Newton and White, 1999).

1.2 The Biology of the Malaria Parasite

1.2.1 The life cycle of the malaria parasite

The life cycle of malaria parasite is comprised of two stages (Figure 2), a sexual phase with multiplication in the female of the *Anopheles* species of mosquito, and an asexual phase with multiplication in a vertebrate host, which in turn has two cycles, one with-in and one outside the erythrocyte. The *Anopheles* mosquito is the predominant vector that can either infect mammals or itself be infected during a blood meal: the mosquito bite. Of the two hosts, humans are the intermediate host and the *Anopheles* mosquito the definitive host, as in it the sexual phase of the cycle occurs (Sherman, 1998). Each species of *Plasmodium* infecting humans exhibits different morphological stages (i.e. sporozoites, merozoites, etc.) that elicit unique host immune responses, and each of these is a potential target for an antimalarial vaccine (Engers and Mattock, 98).

Parasites taken up by the mosquito during a blood meal as gametocytes transform into male exflagellated microgametocytes or female macrogametocytes and are released from the erythrocytes in the midgut of the insect where fertilization occurs, forming the 'zygote' within 18h of the blood meal. The zygote within the midgut then elongates becoming a motile ookinete which enters the midgut epithelium and comes to rest beneath the basal lamina where it forms an oocyst 24-72h after the blood meal. The oocyst then matures in 7-15 days after the blood meal giving rise to up to 10,000 sporozoites. Finally, the sporozoites move through pores (Bannister *et al.*, 2000a) in the oocyst membrane into the haemocoelic fluid to accumulate in the acinal cells of the salivary gland. They have been found to bind specific receptors on the salivary glands as antisporozoite antibodies inhibit salivary gland invasion (James *et al.*, 1999). They also form a vacuolar membrane and appear to form a junction with the epithelial cell membrane at one end (Pimenta *et al.*, 1994). The vacuolar membrane then undergoes lysis to leave the sporozoites unprotected inside the salivary gland. Here they
Figure 2. The life cycles of the malaria parasite. A. Liver Stage: 1. Infective sporozoites are inoculated into the vertebrate host during a blood meal, they, 2. migrate to hepatocytes and, 3. multiply by schizogony until, 4. rupture of host cell frees merozoites into blood stream, B. Blood Stage: 5. Here they infect erythrocytes and, 6. develop through trophozoite stages, either to form, 6. schizonts that rupture releasing more infective merozoites or, 7. differentiate into gametocytes. C. Mosquito Stage: 8. Gametocytes are taken up by the mosquito during a blood meal where they mature into male exflagellated microgametocytes or female macrogametocytes and join, 9. to form zygote that, 10. elongates into a motile oocinete, crosses intestinal epithelium and, 11. forms oocysts beneath the midgut basal lamina. 12. Oocytes rupture when mature liberating up to 10,000 sporozoites that move to salivary gland to complete the parasite life cycle {From Hoffman, 1996}. 
mature into infective sporozoites that are again inoculated into the vertebrate host during the next blood meal.

Sporozoites injected incidentally during the mosquito bite move within minutes to invade hepatocytes if they are not first cleared by the spleen or if pre-existent anti-sporozoite antibodies are not present to impede their progress. They are usually not injected directly into a blood vessel but, as the mosquito searches, it releases vasodilators with the saliva to increase its chances of finding one, thus depositing any sporozoites present into the epithelium (Boyd and Kitchen, 1993). When the mosquito finally finds a blood vessel, it ingests blood, and no longer releases saliva or sporozoites. This was shown when mice that were bitten by mosquitoes on the ears were infected less frequently if the site was excised within five minutes post-inoculation (Sidjanski and Vanderberg, 1997), and was further supported by a demonstration that sporozoites can travel through an epithelial layer in vitro (Mota et al., 2001). Once in the blood stream sporozoites bind to specific receptors on liver cells (Cerami et al., 1992 and Frevert et al., 1993). The sporozoite surface has a co-receptor complex, including the circumsporozoite protein (CSP, Dame et al., 1984) and the thrombospondin related apical protein (TRAP, Robson et al., 1995), which contains related thrombospondin domains. It is the thrombospondin domain that binds specifically to glycoaminoglycan (GAG) chains of heparan sulfate proteoglycans on hepatocytes in the area that is in apposition to the sinusoidal endothelia and the Kupffer cells. In vitro, parasites invade Kupffer cells and enter a vacuole that does not fuse with lysosomes (Pradel and Frever, 2001). In vivo it is believed that sporozoites bind heparan sulfate proteoglycans protruding into the sinusoidal lumen through the fenestrated endothelium and then flow towards the next Kupffer cell, which they pass through in order to gain access to the space of Disse, which is the side of the liver cells exposed to circulation (Chitnis et al., 1999).

In the liver, the exo- or pre-erythrocytic stage is played out over a period of less then a week of asexual divisions in hepatic parenchymal cells (Miller and Hoffman, 1998). The exact duration depends on the size of the fully grown schizont and the number of merozoites it contains, and this depends on the species of the malaria parasite involved. P. falciparum takes a minimum of five and a half days for merogony to be completed. Here, even in their intracellular form, they are liable to attack, this time from cytotoxic cells that target the infected hepatocytes (Good and Doolan, 1999), primarily by CD8+ T cells but also CD4+ T cells. These cells either secrete IFN-γ that induces nitric oxide-dependant killing or destroy the infected cells directly (Miller and Hoffman, 1994). P. vivax, P. ovale, and the simian malaria P. cynomolgi may develop hibernating forms, termed hypnozoites. Surviving, they
will break their containment to release thousands of merozoites through the liver sinusoids and into the blood-stream from each single infected hepatocyte. At this point, there is a way of microscopically differentiating amongst species, as *P. falciparum* will produce about 30,000 mononucleated merozoites per cell, whereas *P. vivax* produces about a third of these, *P. ovale* makes a few more than *P. vivax* and *P. malariae* produces only some 2000 (Barnwell and Galinski, 1998). These free merozoites make a perilous journey, avoiding host defences, in search of a red blood cell to invade. The merozoite (Figure 3) has an elongated structure of various sizes according to the species considered. The smallest, of an approximate length of 1.6 \( \mu m \) and a diameter of 0.7 \( \mu m \) is *P. falciparum*, *P. vivax* has a diameter of 1.2 \( \mu m \) and *P. ovale* is largest at 1.8 \( \mu m \) (Barnwell and Galinski, 1998). The monkey malaria, *P. knowlesi*, was fundamental for the elucidation of the key steps in process of erythrocyte invasion (Figure 4, images 5 to 7). Invasive *P. knowlesi* merozoites can be isolated from infected rhesus monkeys and the invasion process has been filmed by video microscopy and also observed by electron microscopy (Dvorak *et al.*, 1975).

Invasion is a multi-step process (Ward, Chitnis and Miller, 1994) that can be broken down into three major steps (Figure 4, images 1 to 4): 1. Initial attachment and reorientation that is reversible is followed by, 2. junction formation that is irreversible (Figure 4, image 4D), and finally, 3. parasitophorous vacuole formation and entry. Step one begins with an initial contact of the parasite anywhere on the merozoite surface. The parasite can still glide off the host cell at this point so attachment is said to still be reversible at this time. There is a dynamic procedure of interaction among surface coat filaments that is thought to act, by as yet unknown receptor-ligand interactions, as the initial driving force in attachment and reorientation (Berzins, 2002). This coat is then cleaved off as the invasion process proceeds (Aikawa *et al.*, 1978). At the point of contact there is a slight invagination of the erythrocyte membrane (Ladda *et al.*, 1969), then the reorientation of the apical prominence that assures that it is juxtaposed to the erythrocyte before the formation of a tight junction (Aikawa *et al.*, 1978 and 1981), a layer of electron-dense material just under the inner leaflet of the red cell membrane’s bilayer surface (Miller *et al.*, 1979). The merozoite is now commited to invasion. The transient deformations that have already begun on the surface of the red cell become a visible indentation as the apical organs, led by the rhoptries, begin to discharge their contents (Bannister *et al.*, 1986 and Bannister and Mitchell, 1989) and the junction extends itself in a zipper-like fashion around the merozoite as it enters the red cell through the growing indentation. On entering, the parasitophorous vacuole is formed around it (Bannister *et al.*, 1977), and the two junctions continue to move as a circumferential band towards the posterior of the merozoite where they will fuse thus leaving it wholly within the vacuole. In anywhere
Figure 3. *Plasmodium* merozoite. Cut-away model depicts cellular environment of a mature malaria merozoite. Components with proteins involved in invasion are: the dense granules that contain, among others, the PfSUB family of proteins; the rhoptries with the RAP and Rhop complexes; micronemes contain the DBL-EBP family, and; the membrane has the Merozoite Surface Protein (MSP) family, Apical Membrane Antigen-1 (AMA-1), MAEBL (and extended family), *P. falciparum* Reticulocyte Binding Protein homologues (PfRBL), and the Serine Repeat Antigen (SERA) {From Bannister *et al.*, 2000}. 
Figure 4. *Plasmodium* merozoite invasion of erythrocyte. This representation of erythrocyte invasion by malaria merozoite {From Chitnis and Blackman, 2000} shows the major stages as well as important events and key proteins involved. Stage 1. Attachment and reorientation, is still a reversible interaction, 2. Junction formation, attachment is now irreversible, and 3. formation of the parasitophorous membrane that will grow to surround the parasite for its stay within the host cell. 4. Again shows invasion {From Cowman and Crabb, 2002}, this time highlighting parasite/erythrocyte interaction at the tight junction (4D). Electron micrographs show: 5. junction formation, 6. and the parasitophorous membrane forming between the two junctions moving around the parasite until, 7. merozoite is completely enveloped by the erythrocyte {From Dvorak et al., 1975}. 
from 36 to 48 hours from the onset of invasion (Bannister and Mitchell, 1989) the merozoite will multiply by schizogony.

Once invasion is complete and far into the trophozoite stage, the point of entry shows what has been defined as ‘scar tissue’ (Nagao et al., 2000) though others have proposed it to be the terminus of the parasitophorous duct used to exchange molecules with the outside world (Pouvelle et al., 1991). Inside the red cell development continues to the trophozoite stage, and early on they look like rings under a light microscope with Giemsa staining. This part of malaria life cycle is thus called the ‘ring’ stage. Infected erythrocytes become distorted and irregular during the mature trophozoite stage. Protein synthesis increases dramatically as nuclear division begins. Each nucleus will form a new parasite that, upon rupture of the erythrocyte will liberate invasive merozoites into the blood stream. Some parasites will not undergo schizogony but differentiate into sexual stage, extra-cellular, male or female gametocytes (micro- or macro-gametes). The gametocytes are then ingested by the mosquito when it takes it’s fateful blood meal. Though the mature gametocytes are present in the blood of infected vertebrate hosts, mating only occurs in the midgut of female Anopheline mosquitoes. Gametogenesis takes about 20 minutes, beginning immediately after the parasitized RBC reaches the gut. The parasite dissolves the two erythrocyte membranes surrounding it and, in the case of the male gamete, undergoes the nuclear reorganization necessary for the formation of several flagellar, free swimming gametes (Sinden et al., 1996). The signal that activates the pathway leading to gametogenesis, the gamete activation factor, comes when the parasite is in the mosquito midgut (Carter and Nijhout, 1977). The factor is a tryptophan metabolite called xanthurenic acid that could be developed as a target for drugs aimed at impeding the transmission of malaria (Garcia et al., 1997 and 1998).

Within the erythrocyte the parasite ingests hemoglobin for its amino acid needs, and uses the cell’s glucose and other nutrients which are digested inside numerous phagosomes. Its diet becomes its main enemy as toxic heme is released into the cell as a by-product of hemoglobin digestion. This is, however, polymerized into hemozoin, the insoluble malarial pigment that is harmless to the parasite.

The parasitemia increases exponentially as mature schizont-bloated erythrocytes rupture in synchrony releasing 10-30 merozoites each, along with the hemozoin and other by-products of the parasite’s metabolism. It is at this point that the high fever characteristic of malaria can be observed.
In order to attack the malaria parasite we must look to those stages in the life cycle where it is the most susceptible to immune attack (Figure 5), as when it is extracellular rather than intracellular. The extracellular stages, sporozoites, merozoites and gametes express molecules with functional roles in the biology of the parasite where immune effector mechanisms may be most effective, and it is here that one can look for potential vaccine candidates (Good, 1995). Vaccines are also being aimed at the mosquito and liver stages as well, the first would effectively limit areas of contagion, these are also known as transmission-blocking vaccines (TBVs), whereas the second would reduce the probability of disease occurring in the individual. Asexual stage vaccines on the other hand are aimed at the blood stage, the symptom causing stage of malaria, and would reduce the severity of the disease and the risk of death from infection (Miller and Hoffman, 1998 and Carter et al., 2000).

1.2.2. Morphology and characteristics of *Plasmodium* spp.

The phylum Apicomplexa gets its name from the characteristic localization of a set of membrane bound organelles and cytoskeletal structures known as the apical complex. This common feature is found at the apical end of the parasite that leads the entry into the host cell during invasion as it plays a crucial role in the recognition and in the invasion of target cells (Sam-Yellowe, 1996 and Barnwell and Galinski, 1998). Malaria is unique among parasites as it is invasive on more than one occasion, and in more than one organism, so the importance of these organelles is justifiably great for both the parasite and for researchers looking for a source of vaccine candidate antigens. Organelles found in the apical region area are: rhoptries, micronemes, and dense granules. Also of note is the plasma membrane as the merozoite is surrounded by a complex surface of mostly unknown composition, synthesized and assembled in the final stages of merogony and is stripped off as it penetrates past the junction during invasion. Moreover, there is an abundant presence of secreted proteins in the parasitophorous vacuolar space, any number of which may associate with the merozoite surface, but the little knowledge of their composition and the presence of parasite-encoded proteolytic enzymes in the same space add to the difficulty of proposing any coordinated visualization of the invasion process. Parasite-encoded cysteine and serine proteases that direct the progression of the life cycle are also found in the area and are currently being examined to find molecules that specifically inhibit their functions, as these would be useful tools in both research and therapy.
The target is the *Plasmodium* spp. life cycle. 1. During a blood meal, invasive merozoites liberated into peripheral circulation of host first invade hepatocytes then exoerythrocytic schizogony liberates invasive merozoites, 2. into the blood-stream, where they will invade red blood cells. Erythrocytic schizogony liberates merozoites that either reinvoke RBCs or differentiate into gametocytes. 3. The mosquito takes up *Plasmodium* gametocytes in a blood meal from an infected host. A macrogametocyte will fuse with an exflagellated microgametocyte forming a zygote, this fertilized ookinete then enters the midgut and develops into an oocyst that ruptures when mature with sporozoites. These migrate to the salivary glands and are transferred to the host with the next blood meal.

**Vaccines aim to:** 1 Produce antibodies to sporozoites that block hepatocyte invasion, and, in a cellular response, induce both cytotoxic T-cells that can lyse infected hepatocytes, and IFN-γ that can inhibit liver stage development. 2 Reduce the symptom-causing asexual stage with antibodies that block merozoite cytoadherence and/or invasion of RBCs. Antibodies to antigens on parasitized RBC, and induction of IFN-γ and other cytokines would destroy infected RBCs. 3 In the mosquito, vaccine-induced host antibodies taken up with the blood meal can either block sporozoite development or target the vector directly, to limit resistance to drugs and to vaccines, and thus avoid foreseeable epidemics (after Chitnis and Miller, 1994 and Miller and Hoffman, 1998)
Surface proteins show little glycosylation, although the most studied and best characterized of them, merozoite surface protein-1 (MSP-1), is anchored via a glycosylphosphatidyl-inositol (GPI) at the carboxyl terminus. Antibodies to MSP-1, as to other surface proteins all have certain abilities to block invasion, but an important piece of information to be kept in mind in examining all the available results is that there is always the underlying possibility of redundancy in the invasion pathways. The necessary post-translational processing of MSP-1 is complex and essential, thus making it a potential target for inhibition (Cunha et al., 2001).

The rhoptries are extended drop-like organelles present in all members of the phylum only during their motile, invasive stages. They are made up of two distinct parts, an electron-dense rounded basal bulb made up of short fibrils, around 5 nm long and tightly packed, and a less dense tapering duct that empties just beneath the surface of the plasma membrane that covers the apical prominence (Bannister et al., 2000b). They each contain different sets of rhoptry proteins, the bulb housing the RAP complex (RAP 1/2, Crewther et al., 1990) while the duct contains apical membrane antigen-1 (AMA-1), a *P. falciparum* 225 kD rhoptry protein (Roger et al., 1988) and Rhop-1 (and probably the whole Rhop-1 to 3 complex as well) in an excellent example of packing multi-protein complexes in a confined area (Sam-Yellowe et al., 1995). The rhoptries have a typical bilayer membrane the composition of which is varied. Though research has found the membrane to contain proteins known to associate with lipid bilayers such as transmembrane channels, their characteristics still remain unknown (Bannister et al., 2000b).

The rhoptries discharge their contents onto or into the erythrocyte membrane during the sequence of events culminating in invasion. The tips of the ducts fuse together and with the plasma membrane (Aikawa et al., 1978) as they empty, losing their shape as they discharge their contents out of the parasite. Purified fractions of this effluvium or of antibodies raised against one or another of the proteins contained therein have been shown to block invasion (Ridley, 1990). Immunization with native protein (Siddiqui et al., 1987) or with subunit vaccines made of rhoptry proteins (Chang et al., 1996) also protected Aotus monkeys challenged with *P. falciparum* infected erythrocytes. The trafficking of these proteins seems to be important to invasion, for example, *P. falciparum's* apical membrane antigen-1 (AMA-1, homologous to *P. knowlesi*'s Pk66) has been clearly shown to undergo both a change in location and proteolytic processing during invasion. These changes have not yet been assigned a clear functional purpose. Vaccine components based on rhoptry proteins are definitely promising vaccine candidates. In fact monoclonal antibodies to rhoptry proteins
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have been found to inhibit parasite replication in vivo and inhibit merozoite invasion of erythrocytes in vitro (Ridley et al., 1990).

In addition to proteins, the rhoptries store lipids that have also been attributed an accessory role in invasion and/or parasitophorous vacuole (PVM) formation, though lipids for the PVM are also contributed by the host cell (Ward et al., 1993). In fact, it has been calculated that if all the lamellar contents of the rhoptries were transformed into a contiguous membrane, it would be sufficient to create a vacuole large enough to house the merozoite itself (Preiser et al., 2000).

The micronemes are another set of apical protein-rich organelles that discharge their contents at the time of invasion. They are formed by vesicular budding from the Golgi apparatus like the other apical organelles. The size and number of these small elongated sacs vary. In P. falciparum they are about 120 nm in length, and are attached at one end to the rhoptry ducts. They have typical cytoplasmic membranes with a fine granular content that changes according to the stage of development reached by the parasite. They disappear during invasion as their membrane fuses with the rhoptry duct, though they also seem to partially discharge their contents during the rupture of schizonts (Bannister and Mitchell, 1989). It has been found that the erythrocyte binding protein (EBP) family that is implicated in target-cell recognition and binding is localized in micronemes. Logically, it would be expected that they should be expressed on the surface of the merozoite when in proximity to the erythrocyte, yet EBPs have in fact never been demonstrated to be present on the merozoite surface before or during invasion (Adams et al., 1990, and Sim et al., 1990). Micronemes have only been obtained as enriched preparations from other Apicomplexa, and not from Plasmodium. This makes it ever more difficult to make an educated hypothesis as to their function especially in light of their simple but noticeably different composition and quantity between species and from stage to stage within a single species (Preiser et al., 2000).

The third set of organelles that are found in the apical complex are the dense granules. They are small, independent, rounded vesicle-like bodies at the apical end of the merozoite cytoplasm that empty at a much later stage, that is, after invasion is complete, thus spilling their granular contents directly into the parasitophorous vacuole (Bannister LH et al., 2000a).

1.2.3 Special characteristics of P. vivax.

Most morphological interspecies differences between Plasmodium parasites are too sublime for all but highly trained observers (Figure 6). They do however show minor differences in
size of the parasites. *P. vivax* trophozoites are often seen in various stages of development on one slide under examination and double, or mixed infections with *P. falciparum* are not rare (Figure 6D, Arez et al., 2003).

More prominent morphological differences can be seen during the ring stage, when *P. vivax* bears an irregular amoeboid structure until the RBC is nearly full and 'stippled' with Schuffner’s dots (called James dots in *P. ovale*), caveolae-vesicular complexes that stain pink with modified Leishman’s stain. In Giemsa stains, the shape of parasites is often distorted, though in general, the thinner the film, the clearer the image. Too thin a film will make the stippling of the RBC difficult to see, though irregularities in shape are still visible. Still, in order to examine the microscopic characteristics of *Plasmodium vivax*, the classic method of thick smear 2% Giemsa staining (G-TS) remains the standard. Other methods have also become quite common since they are easier to interpret for less specialized operators. A series of fluorochromes are available in systems that offer results that are quick and easy to read (Aslan et al., 2001). Acridine orange is used for the technique called quantitative buffy coat determination (Becton Dickenson, USA). Most of the other systems use immunological capture and detect methods. ParaSight-F™ (Becton Dickenson), ICT Malaria Pf assay, Malaquick™ (both from ICT (Immuino-Chromatographic Tests) Diagnostics, Australia), PATH™ falciparum malaria IC strip (Quorum Diagnostics, Canada) and Determine™ Pf test (Abbott Diagnostics, USA) contain reagents that bind the histidine rich protein-2 (HRP-2) and OptiMAL™ (Flow, Inc., USA) detects *Plasmodium* lactate dehydrogenase.

Most of the systems mentioned above give simple yes/no answers to the question of whether malaria is present, and if so, is it *P. vivax* or *P. falciparum*. In expert hands, or rather, with expert eyes, Giemsa staining is still capable of gaining much more information about the parasite. *P. vivax* trophozoites are very small in an enlarged erythrocyte, though in the case of a *P. ovale* infection the red cell would be easily seen to be elongated in the shape of a comet. As the *P. vivax* trophozoites increase in size the thin single chromatin thickens, and a large vacuole that forms eventually disappears as the nucleus divides and schizonts form. Schizogony in *vivax* lasts about 48 hours and yields 12 to 24 schizonts. The gametocytes are round or oval without a vacuole whereas in *P. falciparum* they have their particular falciform shape (Caramello, 2002).

Apart from these visible points that can be used to identify the invasive species of *Plasmodium*, there is the known preference of *P. vivax* to invade reticulocyte-stage blood cells. There is also the tendency of the hypnozoite stage to occult itself in hepatocytes (Newton and White, 1999), causing occasional out-of-season relapses of blood-stage
Figure 6. Identification of *P. falciparum* and *P. vivax*. Microscopy remains the most reliable method for diagnosis of malaria and for identification of *Plasmodium* species responsible for infection. Morphological changes throughout the life-cycle allow easy discernment between: *A.* *P. falciparum*, *B.* *P. vivax* and other species, such as *P. ovale* that is noticeably smaller than *vivax*. *C.* *P. vivax* schizont (two below) and trophozoite (two above) stages can be seen in these parasitized RBCs, the late ‘rings’ will eventually make their host RBCs take on amoeboid characteristics. *D.* Shows a mixed *P. vivax* and *P. falciparum* infection that is easy to diagnose using figures *A* and *B* {A., B. and D., Caramello, 2002, and C., WHO, 1998}. 
infections. Finally, there is the fact that \textit{P. vivax} remains very difficult to culture. This greatly hampers the capability to evaluate candidate drugs and vaccines, though in vitro methods are constantly being improved in order to the make the most intelligent use of limited animal models used for more advanced stages of drug and vaccine testing.

1.3 Morphology of Erythrocyte Invasion

1.3.1 Invasion of erythrocytes by malaria parasites

The obligatory erythrocytic stage of malaria is mediated by the specific interaction of red cell receptors and parasite ligands (Chitnis, 2001). The steps involved as the merozoite enters the erythrocyte have been described in detail above and graphic representations are shown in Figure 4. Yet these are only superficial observations that can be easily misinterpreted without a clear idea of the underlying molecular interactions involved. Even so, few receptors on the red blood cell are implicated in the invasion process when compared to the many parasite proteins that are now known to be involved. Work on both opened from the mid-seventies, with the application of real-time video (Dvorak, 1975) and electron microscopy (Aikawa, 1978), that allowed a closer look at the morphology of invasion.

It was, however, not by microscopy but through the initial observation of how Duffy-negative human erythrocytes were resistant to \textit{P. knowlesi} invasion (Miller \textit{et al.}, 1975) that it was understood that \textit{P. knowlesi} required the Duffy antigen to invade human red cells. This information was extrapolated to understand the resistance of West Africans, who are predominantly Duffy-negative, to infection with \textit{P. vivax}. In order to test this hypothesis both Duffy-positive and Duffy-negative volunteers were originally infected with blood-stage \textit{vivax} malaria and it was found that only the individuals with Duffy-positive red cells developed blood stage infections (Miller \textit{et al.}, 1976), thus showing an absolute correlation between Duffy-negativity and resistance to \textit{P. vivax}. This also showed that the receptor required for invasion of human erythrocytes by both \textit{P. vivax} and \textit{P. knowlesi} (Miller \textit{et al.}, 1975) was the Duffy blood group antigen.
1.3.2 Erythrocyte receptors

Among the best characterized of erythrocyte receptors is the Duffy blood group determinant used by both *P. vivax* and the simian malaria *P. knowlesi* for the invasion of red blood cells (Miller *et al.*, 1976). It has been demonstrated that when *P. knowlesi* merozoites interact with Duffy-negative human erythrocytes the initial contact and apical reorientation take place, but the tight junction does not form. This indicates that the interaction between the Duffy binding protein and the Duffy antigen is necessary for junction formation, a critical step in the invasion process. The Duffy antigen is a 38 kD glycoprotein (Hadley *et al.*, 1984; Nichols *et al.*, 1987) that contains seven putative transmembrane domains and 66 extra-cellular N'-terminus amino acids (Chaudhri *et al.*, 1993). The binding site for *P. vivax* and *P. knowlesi* has been mapped to a 35-amino acid segment of the extra-cellular region at the N'-terminus of the Duffy antigen (Chitnis *et al.*, 1996).

As its original name suggests, DARC (Duffy Antigen Receptor for Chemokines) is basically a receptor for pro-inflammatory chemokines (Figure 7) amongst which are regulated upon activation normal T cell expressed and secreted (or RANTES), interleukin-8, and melanoma growth stimulatory activity (MGSA, Horuk *et al.*, 1993). The latter two have been shown to inhibit *in vitro* invasion of susceptible, human Duffy positive erythrocytes by *P. knowlesi* even at nanomolar concentrations. This shows that it is conceptually possible to inhibit invasion by blocking the critical junction formation step (Chitnis, 2001).

In addition to the presence of the Duffy antigen on human erythrocytes it is also expressed as a chemokine receptor on other cells. The function of the Duffy antigen as a chemokine receptor when it is expressed on endothelial cells of post capillary venules, and in some epithelial cells, has yet to be determined (Tournamille *et al.*, 1997). There is a mutation underlying the serological differentiation between the Duffy blood groups. The Fy(a/b) polymorphism is due to a single amino acid substitution (G44D), though the absence of the Duffy antigen in Duffy negative individuals is determined at the promoter level by a disruption of the GATA motif that abolishes erythroid expression without effecting any change in expression on other cells (Figure 7, I and II). The Duffy antigen is still expressed on endothelial cells even in Duffy negative individuals. The Duffy negative phenotype is predominant in the West African population and gives them complete protection from *P. vivax* malaria (Miller *et al.*, 1975). It was an interesting and very useful discovery that the treatment of Duffy positive erythrocytes with chymotrypsin removes the Duffy antigen and can be used to mimic Duffy negativity for *in vitro* assays (Camus and Hadley, 1985).
Figure 7. The Duffy blood group determinant, also called DARC (The Duffy Antigen Receptor for Chemokines). Two representative views of the Duffy antigen, I. and II. The *P. vivax and P. knowlesi* Duffy binding protein binds the N’-terminal residues. The polymorphism responsible for Duffy positive/negative subtypes is shown at the sign (*), whereas Duffy negativity is found in the erythroid specific motif GATA(44) at the promoter level, IIIA. *P. vivax* surface proteins serve as immunogens to induce the production of invasion blocking Abs. IIIB. Antibodies against *P. falciparum* variant cytoadherence proteins can block cytoadherence, forcing the parasite to circulate through the spleen, yet antigenic variation allows parasite to escape. Another strategy for vaccine development is to identify domains that are shared among different variants of the cytoadherence protein and use antibodies against them to block cytoadherence in a variant-independent fashion {I. and III. From Hadley and Peiper, 1997, II. From Tournmouille, 1997}. 
Whereas both *P. vivax* and *P. knowlesi* are absolutely dependent on the Duffy antigen to invade human red cells, *P. knowlesi* can also invade its preferred host, rhesus monkey erythrocytes as well as chymotrypsin-treated rhesus erythrocytes that have been stripped of the Duffy antigen and trypsin-treated Duffy-negative human erythrocytes by other, as yet unknown, receptors (Haynes *et al.*, 1988). Moreover, the fact that *P. vivax* preferentially invades reticulocytes implies there is interaction with some unknown receptor on reticulocytes during invasion (Galinski and Barnwell, 1996).

It was originally found that the invasion of erythrocytes was mediated by the interaction of a 175 kD *P. falciparum* erythrocyte binding antigen (EBA-175) with terminal sialic acid (N-acetyneuraminic acid) residues on glycophorin A (GYPA, Camus and Hadley, 1985). Erythrocytes that do not express glycophorin A (En[a-]), but still express glycophorin B and other sialo-glycoproteins, are not bound by EBA-175, thus implying that the glycoprotein backbone structure is also required for binding. EBA-175 belongs to the same family of erythrocyte binding proteins (EBP) which also includes the *P. vivax* Duffy binding protein and three *P. knowlesi* proteins; the α protein, that binds the Duffy antigen on human and rhesus erythrocytes, and its paralogues, the β and γ proteins, that bind Duffy-independent receptors on rhesus erythrocytes (Sim *et al.*, 1990, Adams *et al.*, 1992, Chitnis *et al.*, 1994 and Singh *et al.*, 2002). *P. falciparum*, like *P. knowlesi*, has different, independent pathways available for invasion (Mitchell *et al.*, 1986, Hadley *et al.*, 1987, Perkins and Holt, 1988, Dolan *et al.*, 1994 and Okoyeh *et al.*, 1999). *P. falciparum* uses at least four independent pathways for invasion, using sialic acid residues on glycophorin A (Miller *et al.*, 1977), glycophorin B (Dolan *et al.*, 1994), and glycophorin C (Maier *et al.*, 2003, and Lobo *et al.*, 2003) as well as a still unknown trypsin sensitive and neuraminidase resistant receptor ‘X’ or a trypsin resistant and chymotrypsin sensitive receptor (Gilberger *et al.*, 2003). Under selective pressure it may switch between these different invasion pathways although the molecular basis of such a switch is not yet completely understood. The switch to alternative sialo-glycoproteins for binding was originally demonstrated in some laboratory strains (Dolan *et al.*, 1990). However, it has been shown that invasion by alternate pathways is also a common occurrence in field isolates, and not an artefact of *in vitro* parasite culture (Okoyeh *et al.*, 1999).
1.3.3 Parasite ligands

Merozoite invasion of erythrocytes clearly depends on the ability of the parasite to attach itself to the RBC membrane. The antigens involved are many, in fact, malaria parasites have been rightly called 'a jigsaw with an increasing number of pieces' (Heddini, 2002). Classification is best organised according to their solubility and their localization within the parasite as also by sequence homology. Those localized within the apical organelles function in later stages of invasion whereas those on the surface of the parasite are thought to be involved in the early steps of molecular recognition.

Malaria parasites must make use of the limited set of receptors available on the surface of the erythrocyte. *P. falciparum* is able to invade erythrocytes by selecting diverse receptors and multiple pathways (Miller et al., 1977), thus showing how it is in the best interest of the malaria parasite to be flexible in its choice of site for attachment and invasion. The recent sequencing of the *P. falciparum* genome sequence has made clear that many molecules involved in interactions with the red cell membrane are members of gene families (Gardner et al., 2002 and Bowman et al., 1999) that must be studied in toto in order to best understand their role in invasion pathways and for the induction of anti-parasite invasion blocking immunity (Cowman and Crabb, 2002).

A major component of the merozoite's dense surface coat is the merozoite surface protein -1 (MSP-1). It is the best characterized blood stage antigen and a major target for vaccines (Berzins, 2002). PfMSP-1 is originally a 200 kD precursor that undergoes various stages of processing leading to a 42 kD protein that is bound by a GPI anchor to the merozite membrane, whereas the larger fragments are only non-covalently bound and form a greater MSP-1 complex along with two other peptides of 36 kD (Stafford et al., 1994 and 1996) also known as MSP-636 (Trucco et al., 2001), and 22 kD, known as MSP-722. MSP-722 contains four sites of sequence variation and several predicted helical and two beta elements with no sequence similarity outside the *Plasmodium* databases. MSP-722 is processed to a 19 kD MSP-719. It has been proposed that MSP-722 results from a protease that may also cleave MSP-1 and MSP-6. A *P. yoelii* gene (YM) has been found to have significant homology to MSP-7 (Pachebat et al., 2001).

MSP-142 is again cleaved into a 33 kD and a C'-terminal 19 kD fragment (Holder et al., 1996), a process that is probably related to the removal of the surface coat as the tight junction passes over the merozoite (Aikawa et al., 1978). The MSP-1 complex is released along with MSP-133 whereas MSP-119 remains membrane-bound and enters with the merozoite into the
host cell. This fragment, composed of two epidermal growth factor (EGF)-like domains, is a major vaccine target, since antibodies raised against this fragment are capable of blocking invasion.

MSP-2 is a myristylated glycoprotein that ranges in size from 35 kD to 52 kD and shows a high degree of polymorphism due to blocks of central diverse repeat and non-repeat sequences flanked by highly conserved N’- and C’- terminal sequences. This amount of polymorphism is indicative of the immune pressure to which the antigen is subjected as the probable target for neutralizing immune responses. MSP-4 and MSP-5 are small GPI anchored *P. falciparum* antigens with single EGF-like domains towards their C’-terminii. The genes encoding the two proteins are arranged head to tail adjacent to the gene for MSP-2 on chromosome 2 (Marshall *et al.*, 1997 and 1998).

Rhoptry antigens are mostly non-covalently associated to the high- and low- molecular weight protein complexes called Rhop-H and Rhop-L (Sam-Yellowe, 1996). Rhop-H proteins are located in the electron-lucent duct region of the rhoptries, they are composed of three proteins called Rhop-1 of 140 kD, Rhop-2 of 130 kD, and Rhop-3 of 110 kD of which only the last has been cloned and sequenced (Sam-Yellowe *et al.*, 1995). Its sequence is conserved among various isolates both in overall sequence as well as antigenic epitopes (Brown, 1991), and mouse antibodies to Rhop-H have an inhibitory effect on invasion (Cooper, 1988). Rhop-H has also shown binding to lipid moieties on the inner leaflet of the human RBC membrane and to the surface of mouse red cells. During invasion it appears to spread out along the RBC membrane and also maps to the PVM (Sam-Yellowe *et al.*, 1988 and 1991).

The Rhop-L complex has been mapped to the bulb or the body of the rhoptries, and is made up of the three rhoptry-associated proteins, RAP-1 of 67 kD (processed from an original 86 kD protein to an 82 kD form), RAP-2 of 39 kD and RAP-3 of 37 kD (Howard *et al.*, 1998a). The secondary processing of RAP-1 is associated with merozoite release and involves only a fraction of the RAP-1_{62} produced. Free merozoites have equal amounts of RAP-1_{62} and RAP-1_{67}, though rings have only RAP-1_{62} (Harnyuttanakorn *et al.*, 1992). Of the many alleles of RAP-1 and RAP-2 that have been sequenced only few scattered point mutations have been found, thus revealing a low degree of polymorphism (Howard, 1992, Howard *et al.*, 1998b and Saul *et al.*, 1992). The function of these proteins remains unknown, though their presence among material released during invasion implicates them in that process. Moreover, monoclonal antibodies to RAP-1 (Howard, 1992 and Harnyuttanakorn *et al.*, 1992) and mouse antibodies to RAP-2 (Stowers *et al.*, 1992) have shown to inhibit invasion. However, no changes in growth or invasion phenotype were observed when these genes were disrupted in
P. falciparum, thus indicating that these parasite proteins are redundant for invasion (Baldi et al., 2000).

The micronemes discharge their contents early in invasion. They contain the erythrocyte-binding antigens that are fundamental to the invasion process. The Duffy antigen-binding proteins (DABP) from P. vivax and P. knowlesi (Adams et al., 1992, See Figure 8A) are perhaps the most important of these proteins. They are members of a family of high molecular weight microneme proteins that also includes the P. falciparum erythrocyte binding antigen-175 (EBA-175, Figure 8B). These proteins are collectively known as the Duffy binding-like (DBL) family, and are characterized by a highly conserved cysteine-rich region near the N'-terminal of the molecule called region II (Adams et al., 1992, and Chitnis, 2001). According to the species in question, this region contains from 12 to 14 cysteines involved in intramolecular disulphide bonds. These cysteines are crucial for the correct conformation and activity of the native protein since it has been shown that the EBP binding domain maps to this region. In P. vivax it has been mapped to the 170-amino acid residues found between cysteines 4 and 7 of region II (Ranjan and Chitnis, 1999). In P. falciparum EBA-175 there is a duplication of region II where they called F1 and F2, and only the second (F2) holds the epitope responsible for binding (Sim et al., 1990). EBA-175 is expressed as a 190 kO protein and is processed in the microneme into its 175 kD active form. It has been shown to bind erythrocytes in a sialic acid dependent manner.

DBL domains also mediate cytoadherence as a part of the variant erythrocytic membrane protein-1 (Pf EMP-1, Figure 8D) antigens expressed from var genes on the surface of P. falciparum-infected erythrocytes. In the EBL family of proteins, the conserved cysteine-rich domains take on a critical role, as they recognize specific host cell surface receptors and thus determine the parasite's erythrocyte invasion specificity. These are the products of the erythrocyte-binding-like (ebl) gene family and are among the best-defined ligands from the invasive stages of malaria parasites. In fact, recent work highlights the binding specificities of novel proteins from the erythrocyte binding like (EBL) super-family that include: MAEBL, EBA-181 (JESEBL), EBA-175, EBA-165 (PEBL), and EBA-140 (BAEBL).

EBA-181, also known as JES-EBL, binds erythrocytes in a sialic acid dependent manner, though apparently through neither glycophorin A, B, nor C as the receptor for JESEBL was found to be trypsin resistant and chymotrypsin sensitive (Gilberger et al., 2003). EBA-181 is considered to be functionally equivalent to EBA-175 and EBA-140 (Adams et al., 2001 and Gilberger et al., 2003). Another vaccine candidate, EBA-140 (PfEBP-2 or BAEBL) was found to bind glycophorin C (Lobo et al., 2003 and Maier et al., 2003). BAEBL contains the
Figure 8. Gene structures of Plasmodium spp. erythrocyte binding like (EBL) superfamily. A: 1xDBL domain: P. vivax DBP, P. knowlesi DBP and P. cynomolgi EBP, B: 2xDBL domains: P. falciparum EBA-175, EBL-1, BAEBL, JESEBL, and PEBL, and P. reichenowi EBP, C: maeb: P. berghei, cynomolgi, falciparum, knowlesi, vivax and yoelii MAEBL, D: The var superfamily: P. falciparum EMP-1, E: ama-1: P. berghei, chaubaudi, cynomolgi, falciparum, fragile, knowlesi, reichenowi, vivax and yoelii, (and Toxoplasma gondii) AMA-1. {after Chitnis and Miller, 1994 and Michon et al., 2002}. 
characteristic cysteine-rich motif and neuraminidase treatment abolishes binding to red cells. Trypsin treatment of RBCs reduces 2-fold the binding of BAEBL and 10-fold that of EBA-175. Gerbich negative individuals (they lack the GYPD and have an altered GYPC without the third exon) are in fact less susceptible to invasion (Lobo et al., 2003), suggesting that \textit{P. falciparum} has evolved multiple invasion pathways dependant on polymorphisms in the BAEBL ligand that allow for binding to other receptors (Mayer et al., 2002).

MAEBL and EBA-165 (PEBL) are odd members of this family, related by gene structure and similar C'-terminal cysteine-rich regions (region VI). PEBL has been defined by authors as a "pseudo-gene," and is no longer being examined (Triglia et al., 2001). MAEBL is from a family of rhoptry-associated proteins with a homology to the DBL-EBL family of merozoite proteins (Kappe et al., 1997 and Ghai et al., 2002). The protein is chimeric in nature. The N'-terminal erythrocyte-binding cysteine-rich domains (M1 and M2) characteristic of this family of proteins are similar to subdomains 1 and 2 of PfAMA-1, and the C'-terminal cysteine-rich domain is homologous to region VI of EBPs (Kappe et al., 1998 and Noe and Adams, 1998). MAEBL was identified in \textit{P. berghei} and \textit{P. yoelii}, and in \textit{P. falciparum} the gene maebl was found to be single copy whose gene product localizes to the merozoite apical complex and is also found processed in culture supernatant. MAEBL was identified on the surface of mature merozoites prior to apical reorientation, thus implicating it in an important role during the initial contact of the merozoite with the RBC (Noe et al., 2000).

The ability to invade using alternative receptors is dependent on a parasite's ability to express different types of functionally equivalent ligands. Different \textit{Plasmodium} species within humans and other hosts have evolved distinct invasion pathways to utilize unique sets of erythrocyte receptors. Some species, like \textit{Plasmodium falciparum} and \textit{P. yoelii}, can use multiple alternative pathways of invasion, whereas other species, like \textit{P. vivax}, are dependent on a single receptor during the critical junction-forming step, early in the process of invasion. These parasite ligands can be grouped in the DBL-EBP superfamily that includes the \textit{P. viviax} and \textit{P. knowlesi} Duffy-binding proteins, the \textit{P. knowlesi} \(\beta\) and \(\gamma\) proteins, PfEBA-175 and MAEBL (another promising vaccine candidate against \textit{P. falciparum} malaria).

The \textit{Plasmodium vivax} reticulocyte binding proteins (PvRBP-1 and PvRBP-2) are atypical microneme proteins. They have been localized to the apical surface of merozoites and seem to be responsible for the preference of \textit{P. vivax} to invade only reticulocytes though receptors for them have yet to be identified (Galinski et al., 1992). Homologous genes have been found in \textit{P. cynomolgi}, another parasite that is restricted to the invasion of reticulocytes. (Warren et al., 1966). PvRBP-2 shares homology to the p235 family of \textit{P. yoelii} rhoptry proteins (Keen et al., 2002).
1994) and to *P. falciparum* orthologs PfRBP-2-Ha and -Hb proteins (Rayner *et al.*, 2000). It is interesting to note that the passive transfer of monoclonal antibodies from mice immunized with the *P. yoelii* p235 limits an infection to reticulocytes and protects mice from death when challenged with a lethal strain of *P. yoelii*. This suggests a role for the greater RBP family in erythrocyte invasion (Freeman *et al.*, 1980, and Holder and Freeman, 1981). In fact, the *P. yoelii* p235 undergoes a novel clonal variation of invasion phenotypes where transcription at the single cell level indicates that each merozoite emerging from a schizont expresses a unique p235 gene. Merozoites coming from a single schizont can each transcribe their own different p235 gene, each with unique antigenicity, receptor specificity and invasion phenotype that may allow individual merozoites to escape host immune responses specific for some p235 proteins but not others (Preiser *et al.*, 1999).

Once the parasite has invaded the red cell it discharges the contents of the dense granules. Few antigens have been localized to this site, and even if they are released very late, two have been implicated in invasion: the *P. falciparum* 155/ring-stage erythrocyte surface antigen, called RESA (Aikawa *et al.*, 1990 and Culvenor *et al.*, 1991), and two subtilisin-like proteins PfSUB-1 and PfSUB-2 (Barale *et al.*, 1999, Blackman *et al.*, 1998, and Hackett *et al.*, 1999). Antibodies to RESA repeat regions are effective in blocking invasion and growth, either by entering the parasitophorous vacuole with the merozoite or through a putative parasitophorous duct. Like other soluble antigens, RESA is thought to associate with the merozoite surface although its precise function is not yet known.

The ring-stage membrane antigen, or RIMA, is also present in the dense granules (Trager *et al.*, 1992), but there is no further data on the activity of this or other dense granule proteins, apart from PfSUB-2 that has thus far only been assumed to be responsible for the conversion of MSP-142 to MSP-119 (Barale *et al.*, 1999).

Many malaria antigens are expressed throughout the lifecycle of the malaria parasite. One such antigen from *P. falciparum* is the glutamate rich protein (GLURP). It has a molecular weight of 220 kD. Antibodies in sera of individuals from endemic regions were shown to be directed against GLURP, thus raising hopes of a possible future *P. falciparum* vaccine utilizing GLURP epitopes (Dziegieł, *et al.*, 1991, and Theisen *et al.*, 2001).
1.4 Vaccine Development

1.4.1 Concepts and approaches for the development of malaria vaccines

Resistance of Plasmodium to antimalarial drugs and of the Anopheline vectors to insecticides is a major problem in malaria control. The alternative that has long been the focus of research throughout the world is the development of a vaccine against malaria. The goal of any malaria vaccine is to enhance natural responses, with long-lasting complete protection in both endemic and non-endemic areas (Saul, 1987). This would have to take into consideration the complex life cycle of the malaria parasite, since it passes through various immunologic "compartments" during different stages of life. Until now single proteins or peptides that have been examined as potential agents for vaccination have been shown to be at most partially protective whether in animal or human studies (Holder, 1999). The need is to look towards a multi component vaccine that combines antigens taken from the most vulnerable of these stages. The complexity of the "vaccine solution" increases once the variety of different Plasmodium species that may inhabit any one endemic region is considered. Thus far it has proved to be a very difficult task to produce an effective, long lasting vaccine and there still remains no commercially available malaria vaccine.

The possibility of developing a malaria vaccine still holds much promise, as the proof of concept has been demonstrated in many ways, primarily by showing that immunity to malaria is possible. One observation is based on the fact that individuals continually exposed to infection eventually develop natural immunity. Moreover, passive transfer of purified IgG from immune adult donors exposed to malaria to non-immune patients with acute P. falciparum infections led to a significant reduction in parasitemia (Cohen et al., 1961 and Sabchareon et al., 1991). Finally, immunization with irradiated sporozoites in humans (Rieckmann et al., 1979), and of blood-stage parasites in monkeys (Miller and Hofmann, 1998) provided protection against P. falciparum. It has also been understood that antibodies contribute only partially to immunity. In future, it has been suggested that the direction for research is to integrate those antigens that stimulate protective T cells in the absence of antibodies in order to achieve complete protection from malaria (Good, 2001).

With an eye to these results, the best strategy may well be a combination of the principal targets for a malaria vaccine; the pre-erythrocytic stage sporozoite, and the blood stage merozoite (Shann and Steinhoff, 1999). The former has already shown itself to be effective (Clyde, 1975, Rieckman et al., 1979, Nussenzweig et al., 1967 and Nussenzweig and
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Nussenzweig, 1989). The latter has many advantages as in the free form blood-borne merozoites that are a target for humoral response. Antibodies against surface molecules can agglutinate, aid opsonization, mediate complement driven lysis and interfere with erythrocyte invasion (William and Hoffman, 1999). In order to raise potent antibodies, valid targets must be found. The search for vaccine candidate antigens and their genes has progressed steadily over the years, so that the hundred or fewer genes that were available in the DNA sequence database by the end of the 1980's had grown by the end of the 1990's to nearly four thousand sequences available for scrutiny. With the recently published sequences related to malaria, the human, Plasmodium falciparum and Anopheline mosquito genomes, the number of specific targets is sure to rise.

The principal drawbacks for the development of a malaria vaccine can be found in the parasite’s ability to evade immune detection. The various Plasmodium species, as well as the different stages in the life cycle, do this by either sequestering to vascular endothelium or by invading cells that have a low immune profile. Merozoites invade immunologically invisible erythrocytes, as these cells do not express the major histocompatibility complex (MHC). Hypnozoites can select to remain hidden in hepatocytes. A strategy for vaccine development would use specific parasite ligands as immunogens to induce invasion blocking antibodies (Figure 7, IIIA).

There are many ways to approach the construction of a malaria vaccine by targeting the different stages of the parasite life cycle (Figure 5). The life cycle can be considered to begin in the human host with the subcutaneous injection of sporozoites by an infected female Anopheles mosquito. As the sporozoites invade hepatocytes the pre-erythrocytic stage of the disease begins. Cellular mediated responses (Good, 1999) as well as high titer antibody responses against sporozoite surface antigens (Marussig et al., 1997) are currently the most promising vaccine strategies aimed against the pre-erythrocytic stage.

Once hepatic shizogeny is complete, a host of merozoites invades the blood-stream and begins invading erythrocytes. Blood stage vaccines aim to prevent merozoites from initiating the chain of events that lead to the symptom causing stage of malaria. As merozoites enter red blood cells and multiply, they express proteins on the erythrocyte surface. Vaccines may elicit antibodies against parasite antigens expressed on the surface of erythrocytes. A vaccine that aims to reduce the parasite burden at this point would have the advantage of hitting at the symptom causing stage of the disease, thus reducing the strain on the individual infected with non-lethal species of Plasmodium and reducing mortality in those areas endemic for P. falciparum.
The mosquito stage, also called the sexual stages, offers targets for vaccines that have been deemed Transmission-blocking vaccines (TBVs). These vaccines develop antibodies that are ingested along with the blood meal and are aimed against those antigens expressed on the sexual stages of the parasite, thus blocking the parasite life cycle within the mosquito (Kaslow, 1993). Target groups for the TBVs come from geographically isolated areas with a seasonal or otherwise low-level transmission. There would have to be different components according to location and to the level of endemic disease transmission. It is thought that TBVs could be used as a component of a multi-stage vaccine, or deployed to prevent the spread of malaria strains resistant to protective malaria vaccines. Likewise it could be useful for the containment of epidemic outbreaks of malaria. The difficulties in deploying the vaccine would necessitate a concerted effort between health agencies and local governments in order to achieve optimum levels of coverage (Carter et al., 2000). This may be a challenge greater than that of developing the vaccine itself.

1.4.2 Status of vaccine and development efforts

The present situation is charged with a number of vaccines in various phases of development. The stages in the malaria life cycle against which they are aimed, and that were presented in Figure 5, are the pre-erythrocytic or liver stage, the blood stage, and the mosquito stage. In giving a panorama of individual vaccine candidates that are currently under development it is important to bear in mind that they will, in all likelihood, be employed in a future combination vaccine. The components will be chosen according to the combination that achieves the best protection against malaria. Work is also routed to novel strategies in vaccine delivery, such as new and enhanced adjuvants, live vectors that incorporate antigenic gene sequences and DNA vaccines (Enger and Mattock, 98).

Pre-erythrocytic stage vaccines are focusing on antigens from the sporozoite surface and proteins expressed in infected hepatocytes to retrieve promising candidates. The circumsporozoite protein (CSP) and thrombospondin related adhesive protein (TRAP) as well as liver stage antigens 1 and 3 (LSA-1 and LSA-3) are the most promising of these components. It was demonstrated that irradiated sporozoites were excellent material for a malaria vaccine, and a vaccine was assembled using the circumsporozoite (CS) protein (Ballou et al., 1987 and Herrington et al., 1987). However, clinical trials using the CS protein alone didn’t show the protection that was expected (Herrington et al., 1992). Recently, a switch from the original alum adjuvant to a more recent adjuvant and immune-enhancer,
along with the addition of an immunogenic fusion partner, namely the S antigen from hepatitis B (HbSAg), has caused the CS protein to be re-evaluated as a component in a vaccine against malaria. In fact, as RTS,S the CS protein is now showing great promise in trials in Africa (Stoute et al., 1997). A recent example of trials in the Gambia are with RTS,S formulated in AS02. RTS,S is derived from a recombinant chimeric virus-like particle of HbSAg and HbSAg fused to a *P. falciparum* fragment of CSP. This is seen as the first vaccine that has convincingly shown that humans can be protected against malaria. It is viewed as a promising candidate since protection is not relegated to only one strain (Alloueche et al., 2003), though it is still only a starting point for future combinations as protection is thus far incomplete and short-lived (Bojang et al., 2001).

A formulation consisting of *P. falciparum* CSP repeats is being tested as a fusion partner with recombinant Hepatitis B core protein particles in animals. Promising results from the highly immunogenic viral particles elicited high antibody titer against the B cell epitopes from CSP repeats. A formulation of CSP and Hepatitis B core particles in alum is being tested for safety and immunogenicity in Phase I trials in healthy adults (Birkett et al., 2002). Another formulation of multiple antigenic peptides (MAP) from CSP has also been used to elicit high antibody titer to *P. falciparum* sporozoites. Alum and QS21 adjuvants are being used to test the synthetic MAP vaccine in Phase I trials in humans (Kublin et al., 2002).

An innovative approach to vaccine development came with the development of DNA vaccines. A DNA vaccine utilizes the specific gene or genes of interest cloned into a bacterial plasmid optimized for eukaryotic expression. They have the basic features of any expression plasmid, such as a strong promoter, usually derived from cytomegalovirus, bacterial and eukaryotic origins of replication and resistance markers. They also contain a specific nucleotide motif consisting of an unmethylated cytosine-phosphate-guanosine (CpG) dinucleotide with an optimal flanking sequence of two 5' purines and two 3' pyrimidines (Krieg, et al., 1995) that has the vaccine enhancing effect of stimulating multiple immune cells: monocytes, macrophages, dendritic cells, B cells and T cells. Antigen presenting cells, such as dendritic cells, are also induced to secrete cytokines such as IL-12, TNF-α and INF-α.

Naked DNA vaccines are being used against liver stage parasites to elicit CD8+ T lymphocytes which are critical effectors in protection against malaria in animal models. (Good et al., 1988). Attenuated poxvirus was used to deliver seven malaria antigens, including CSP and other liver-stage antigens, and showed some promise (Tine et al., 1996). Prime-boost regimens using naked DNA plasmids, recombinant fowlpox virus (FPV) or modified vaccinia virus (MVA) that express multiple T cell epitopes (ME) from *P. falciparum*
antigens fused to PfTRAP have all seen success at eliciting a cellular immune response against pre-erythrocytic stage parasites (Schneider et al., 1999).

Finally, DNA plasmids offer the opportunity to express antigens from multiple stages of the malaria parasite. Unfortunately, though DNA vaccines work for mice, they need optimization to work in primates and, of course, in humans. Even without this enhancement of cellular response DNA vaccines can still provide antigens through the MHC class I pathway, as a low level priming of the cellular immune responses (Gurunathan et al., 2000a).

DNA vaccines preferentially elicit a Th1 immune response in mice, this is advantageous to malaria therapy as IFN-γ is critical in mediating protective immunity against the liver stage (Gurunathan et al., 2000b). A recent study showed how DNA vaccination is feasible in humans, as a single-gene P. falciparum CS protein DNA vaccine showed itself to be safe and immunogenic in phase 1 trials (Le et al., 2000 and Wang et al., 1998).

The disease bearing stage of malaria, the blood stage, is a central point of interest in the development of a vaccine against malaria. Of primary interest are the erythrocyte binding proteins (EBPs) such as P. falciparum EBA-175 and P. vivax Duffy binding protein (PvDBP). Both recombinant PfF2 and PvRII have shown to be immunogenic in animal models, and to elicit high titer antibodies that are capable of blocking, in vitro, binding and invasion. Moreover, in a study carried out in New Guinea, PvDBP was found to stimulate lymphocytes to produce INF-γ and IL-10 in infected / exposed subjects (Xainli et al., 2002).

One of the first trials of antigenic synthetic peptides against the blood stage of malaria is represented by Spf66. Developed in South America, it was developed well over a decade ago having had its first successful field trial in the early nineties (Amador et al., 1992). A multi-epitope and multi stage vaccine, it was based on three blood stage epitopes (a 45 amino acids monomer polymerized into a larger molecule) linked by a immunodominant pre-erythrocytic epitope (NANP). Interest in Spf66 waned after it failed to meet expectation in human trials conducted in Thailand (Nosten, 1996) and the Gambia (Bojang et al., 2001).

On the more positive side, in Phase I trials, long synthetic peptides based on B and T cell epitopes in P. falciparum MSP-3 and P. falciparum GLURP have shown to be able to induce cytophilic antibodies that could clear parasites by antibody induced cellular inhibition (Theisen et al., 2001).

Other blood stage vaccines that are currently being tested are based on merozoite antigens such as MSP-1, MSP-2, MSP-4, and MSP-5 and AMA-1. Animal trials have shown that immunization with these antigens provide protection against a successive blood-stage
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challenge. A human vaccine trial of three recombinant asexual blood stage *P. falciparum* antigens corresponding to parts of MSP-1, MSP-2 and *P. falciparum* ring-stage erythrocyte surface antigen (RESA) was completed in Australia, by the SARAMANEI Hofmann-La Roche group, using an oil based adjuvant, Montanide ISA 720 (Saul et al., 1999 and Lawrence et al., 2000). A subsequent study in Papua New Guinea demonstrated safety and immunogenicity in immune adults from an endemic area (Genton and Corradin, 2002, and Genton et al., 2000 and 2002). Moreover, the 19 kD C'-terminal processed fragment of *P. falciparum* MSP-1 plays a crucial role in the RBC invasion process and the induction of protective immunity in murine malaria models (Holder, 1996). Work at the Walter Reed army research center has developed *E. coli* derived clinical grade MSP-142 C'-terminal antigen has been produced and is being tested in Phase 1 safety and immunological trials.

Study of the mosquito stage, where the idea is to reduce the chances of contact between the vector and the human host, has produced many potential targets that aim at the parasite’s sexual stages. The most promising of these vaccine candidates are Pfs25 / Pvs25 (Kaslow et al., 1993) and Pvs28 (Miles et al., 2002 and Tsuboi et al., 2003). These are the only ones to go to clinical trials thus far, and have successfully shown the ability to block parasite infectivity to the mosquito vector, thus blocking the spread of the parasite between humans. With wide enough coverage, vaccines with these components could reduce transmission of the disease in endemic regions by reducing the number of infected mosquitoes, and with this, also reduce the heavy burden of malaria on the people indigenous to those regions.

Of the other lead candidate antigens from *Plasmodium* there are Pfs28 (Carter et al., 2000), as well as Pfs230 and Pfs48/45 (Duffy and Kaslow, 1997). These have shown to have some effective transmission blocking activity. Experiments have shown that only one of the two zygote surface proteins (the Pvs25/Pvs28 from *P. vivax* and Pfs25/Pfs28 proteins from *P. falciparum*) can be knocked out without being lethal to the parasite. Therefore by associating these two into a single vaccine there may be a synergism in inducing transmission blocking antibodies. Antibodies against Pfs48/45 cause a significant reduction in transmission due to the inhibition of zygote development, and therefore of mosquito infection (Vermeulen et al., 1986), though all that is known about this protein is that it is specifically expressed in gametocytes and is thought to be anchored to the parasite surface by a GPI linkage.

Pfs25 is the leading *P. falciparum* TBV candidate and it has already entered clinical trials for safety and immunogenicity as an alum formulation in the USA. The vivax homologue that is being prepared as Pvs25H-A is the only TBV against *P. vivax* that is in an advanced stage of preparation. Antibodies against Pvs25H-A block transmission of *P. vivax* to mosquitoes
(Hisaeda et al., 2000). Recombinant Pvs25 is currently undergoing Phase I clinical trials in the USA.

With all these possible combinations available, the approach of developing a multi-stage, multi-component vaccine is another viable alternative. NYVAC-Pf 7 is a genetically engineered, attenuated vaccinia virus, multi-stage, multi-componant P. falciparum vaccine that includes a transmission-blocking vaccine candidate Pfs-25 together with six additional leading candidate antigens: three pre-erythrocytic proteins: CS, CSP-2 & TRAP and three asexual blood stage antigen: MSP-1, AMA-1 & RESA (Tine et al., 1996). This viral vector vaccine is also being used in combination with Pfs25 for a combined study on priming boost (Kaslow, 1997).

In conclusion, we must remember that any vaccine is formulated with one of many adjuvants such as the saponine based QS21 and one or more immuno-enhancers, like monophosphoryl A. In order to assure a long lasting protective immunity it has been seen that these can be interchanged to modulate the level of protection as was shown with CSP (Stoute et al., 1997). Recent pharmaceutical findings in the field of encapsulation and slow or retarded release particles may also aid the search for a malaria vaccine that insures a continual presence of protective antibodies in the blood stream.

1.4.3 Resistance to malaria: genetic, natural and acquired immunity

There are many genetic mechanisms that lead to natural resistance to malaria. These defense mechanisms come into play at various stages of infection according to genetic differences in the make-up of the host. Erythrocyte invasion can be disrupted if the red cell lacks those components that are necessary for the merozoite to enter, the clearest example are Duffy blood group negative individuals, who are resistant to P. vivax malaria. The selective pressure of malaria has clearly been so strong as to have created these examples of genetic variation in the human population. Whether an individual expresses the Duffy blood group determinant or not, is not a cause for an intrinsic pathology. In α- and β- thalassemia, however, individuals are already paying the price of survival by being less susceptible to malaria at the cost of living with an inconvenient disease. In west Africa, sickle-cell anaemia is another example where natural selection makes one in four children of heterozygous parents succumb to the disease, another one to probably succumb to malaria, but allowing the two heterozygotes to survive since the parasites cannot multiply efficiently. These polymorphisms have also been found in New Guinea and Polynesia (Flint et al., 1986). Other common erythrocyte
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polymorphisms including glucose-6-phosphate dehydrogenase deficiency, and polymorphisms in red cell antigens reflect selection by malaria (Miller, 1994). These strategies to evade infection benefit only the single individual and, much in the same way as immunity to malaria, have no effect on the community as a whole (Table 1).

Natural immunity to malaria comes only after several years of recurring infections (Trape et al., 1994 and Baird et al., 1991) and is only partial, leading to milder or asymptomatic infections (Butcher, 1989). Infection gives a very considerable antigenic challenge to the host immune system, and humans have only an incomplete immunity to the parasite that rapidly disappears in the absence of reinfection. This acquired immunity is seen when adults from endemic regions have few symptoms from intermittent relapses in parasitaemia though children are heavily infected and suffer extensively from clinical disease. However, infants less than three months old are relatively resistant to infection due to the transfer of maternal antibodies and to the presence of fetal hemoglobin F that provides some resistance to growth of malaria parasites (Pasvol et al., 1978).

1.5 Recombinant Protein Expression Methods

1.5.1 Review of methods available for expressing malaria proteins

Synthesis and purification of recombinant Plasmodium proteins is difficult, but advances in bacterial, yeast, and baculovirus expression systems and the addition of tags for purification have yielded several of these proteins in forms pure enough for immunization and structural studies. Regions of MSP1 have been produced in bacteria, yeast, and baculovirus (Burghaus et al., 1994, Chang et al., 1992, Kaslow et al., 1994, and Murphy et al., 1990). An N-terminal fragment of the SERA protein was made in yeast, and recently fragments of the SERA protein have been made in bacteria by using synthetic genes designed to mimic E. coli codon usage (Inselburg et al., 1991 and Sugiyama et al., 1996). AMA-1 has been expressed in and purified from baculovirus-infected insect cells (Narum et al., 1993) as has EBA-175 region II (Daugherty et al., 1997).

The classic method of choice for expression of malaria proteins is Escherichia coli (E. coli). However, the malaria parasite proteins undergo post-translational modifications, the formation of di-sulfide bonds in particular, that differentiate parasite produced native proteins
| Hemoglobin mutations | a. “Sickling” diseases (thalassemias and sickle cell anemia, hemoglobin “S”).  
- Mutations usually monofactorial  
- Confer severe disease phenotypes.  

b. Hemoglobin varieties (C, and E),  
- Geographic distribution in malaria endemic regions,  
- Mild disease phenotypes.  

c. Infant hemoglobin (F),  
- Globin chains normally differ from adult humans, fetus has $\alpha_2\gamma_2$ instead of $\alpha_2\beta_2$.  

d. Glucose-6-phosphate dehydrogenase deficiency,  
- X-link trait has many different mutations,  
- Female homozygotes and male hemizygotes have some protection from malaria.  

| Human Leucocyte Antigen (HLA) | - HLA-B53 (class I restricted CD8+ T cells against parasitized hepatocytes)  
- HLA variation and antigen density are high,  
- no class I expression on erythrocytes.  

| Cytokines/Immune response genes | a. DARC (Duffy positive/negative),  
- P. vivax dependant on this antigen for invasion of RBCs.  

b. TNF mediates fever in malaria,  
- TNF levels increase with malaria severity,  
- mutations in TNF gene increase risk of death from severe malaria,  

c. CD31/PECAM-1 binds cysteine-rich CIDR1α,  
- also ICAM-1, VCAM, MBP, iNOS, IL-1 and ELAM-1 and CSA influence parasite/host cell adhesion in cerebral malaria.  

d. CD35/CR-1 binds EMP,  
- responsible for rosetting,  
- variant encoding SI(a-) antigen is protective.  


Table 1: Resistance to malaria: host factors.
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from bacterially produced proteins. Therefore proteins with complex tertiary structure, such as those malaria antigens with a number of cysteine residues, will usually fold incorrectly and accumulate in inclusion bodies. This can be overcome only through extensive work aimed at developing methods to refold and purify each protein according to its own individual chemical properties (Singh et al., 2001 and Pandey et al., 2002). Eukaryotic expression systems are reasonable alternatives, though each has its own shortcomings. Mammalian expression has shown itself to be perfectly feasible though yields that were obtained in COS7 cells were so low that they needed radio-labeling to develop functional assays using the expressed recombinant protein (Ranjan and Chitnis, 1999). On the other hand, yeasts offer excellent yield, though in order to obtain the desired protein it may be necessary to prepare a synthetic gene that takes into consideration the different codon preference of this expression system.

1.5.2 Baculovirus expression vector system

The baculovirus (BV) expression vector system (BEVS) has become one of the most important systems for eukaryotic gene expression and for the production of heterologous proteins (Figure 9). It is a versatile and easy to use system that offers many advantages over prokaryotic and other eukaryotic systems, such as; it can provide correctly folded recombinant protein with disulfide bond formation, oligomerization and other post-translational modifications such as phosphorylation and glycosylation. Protein can be compartmentalized as required, whether it is to be secreted or withheld in the cell cytoplasm, membrane-bound or within the nucleus (Possee, 1997). Potentially critical differences in processing have thus far limited their use as therapeutic proteins, but BEVS derived proteins are already being employed in diagnostic assays, structure-function relationship studies and in vaccines since they are usually produced in forms that are active and easy to purify (see the Becton Dickenson (BD), PharMingen BEVS manual for an exhaustive list). Moreover, the high yield of secreted protein (up to 100 mg per 10 g of insect cells as reported in the PharMingen BEVS manual) is feasible due to the strong BV polyhedrin promoter, and research is continuing in order to optimize the secretory pathway and to limit interference caused by host (insect) cell functions by co-expressing cooperative elements (Ailor and Betenbaugh, 1999). In particular, when there is production of high levels of recombinant protein with the strong polyhedrin promoter, and the subsequent stoppage of host cell protein synthesis, there is deficit of secretory assistance factors (Hsu et al., 1994 and Hsu and Betenbaugh 1997). This can lead to incorrect secretory processing, especially several days post-infection when the host cell is no
Clone gene of interest into BV transfer vector

Check by RD or PCR & prepare sterile plasmid DNA (10μg/well)

Co-transfect Insect cells with plasmid DNA + AcNPV DNA

Establish insect cell (Sf) culture

Maintain monolayer or suspension culture

Experimental

plasmid DNA only

(see 6b)

miniprep DNA with gene of interest

PA on transf. mix, pick plaques & elute plaques singly

Viruses from single plaques amplified/titred by EPDA

Infect cells, MOI>10 expression test for best protein producing virus

Scale-up expression once enough high titre virus is prepared

Reamplify to get high titre 2nd Passage Stock

Figure 9. Baculovirus expression vector system
Figure 9. Baculovirus expression vector system. The preparation of polyhedrin promoter driven expression of heterologous proteins in insect cells follows a standard protocol:

1: the gene of interest is cloned into MCS of desired baculovirus transfer vector and,
2: verified by restriction digestion or PCR. Alkaline lysis DNA isolation (miniprep) and ethanol ppt are needed to obtain pure and sterile plasmid DNA, which is then,
3: mixed with replication deficient viral DNA (AcNPV) and transfection buffers.
4: Cells revived from frozen stock and working insect cell line (here it is adapted to SFM) is established,
5: maintained as either monolayer or suspension culture, after first few passages extra cells are frozen as they have an ideal working life of 20-30 passages.
6: Transfection mix from 3 is made to cover a 50-70% confluent insect cell monolayer for 5h. Media is then replaced with fresh serum free medium (SFM) and left 4-5 days to incubate at 27°C. Experimental well will have rounded, bloated cells, and control well cells will be small, compact and flattened at points of contact {Photos from PharMingen, Baculovirus Expression Manual, 2001}.
6b: Colour-coded model of co-transfection, both DNAs have sequences flanking the cloning region for the double homologous recombination event that occurs within the Sf nucleus. Recombinant virus is then made to either reinfect other cells in order to increase titre or to produces recombinant protein that can be made to secrete into the supernatant for ease of purification.
7: Transfection mix is collected for plaque assay (PA) to select plaques grown from single viruses, the virus containing agarose is eluted and this,
8: is amplified and the titre is measured by end point dilution assay (EPDA).
9: Small-scale infections with individual viral plaque picks are prepared to test for highest protein producing virus, this is done with high multiplicity of infection (MOI) in order to ensure that all cells are infected, whereas,
10: amplification to obtain the 1st Passage Stock virus is done at low MOI to ensure against contamination, and
11: repeated to get higher titre 2nd Passage Stock virus (preferably by EPDA that allows an accurate quantification of viral titre).
12: Expression is scaled up once a sufficient amount of high titre virus is made.
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longer producing its own post-translational processing apparatus. Therefore, research aims to overcome these limiting factors for a superior yield in the baculovirus-insect cell system. The points being examined would improve the secretory and post-translational machinery by overexpressing those heterologous proteins that are involved in these processing steps (Ailor and Betenbaugh, 1999).

Baculoviruses are from the Baculoviridae family of double stranded DNA viruses that can propagate only in their invertebrate hosts (O'Reilly, 1992). The life cycle of Autographa californica nuclear polyhedrosis virus (AcNPV) begins when the virus is taken up by the insect host cells either by fusion or facilitated endocytosis. Uncoating occurs in the nucleus and DNA replication starts 6 hours post-infection (p.i.). The viral DNA used in BEVS is derived from AcNPV where one or more nonessential genes may be replaced by the gene to be expressed. Amongst these is the polyhedrin gene that produces the 29 kD protein polyhedrin very late in infection. This is the substance that makes up the virus occlusion body matrix within the nucleus. Whereas in the early stages of infection the virus produced buds off from the cell membrane, late in wild type infection the cell bursts under pressure of accumulated virus particles and polyhedrin-based matrix. These occluded viruses are liberated during the final lysis stage of in vivo infection, about six days post-infection. The BEVS takes advantage of the fact that polyhedrin is not an essential protein yet it accumulates to account for up to 50% of the total insect protein. When this gene is replaced by a heterologous gene the recombinant baculovirus becomes occlusion body-negative and is easily distinguished from wild-type viruses. The late stage of infection no longer leads to lysis of the insect cells but to the expression of large amounts of the protein of interest, that can be collected from the supernatant in case the vector contains a secretory signal sequence. Moreover, recombinant viruses will both produce protein as well as re-infect other insect cells. Of course lysis will occur if infected cells are left to die, thus liberating damaging proteases into the supernatant. It is therefore very important to harvest the recombinant protein at the appropriate time.

For ease of handling, heterologous genes are cloned into smaller transfer vectors (such as the 9.7kb pAcGP67B secretory vector used here) that are then co-transfected into Spodoptera frugiperda (Sf) cells with linearized AcNPV DNA engineered with a lethal deletion (such as the Baculo-Gold™ from B.D., PharMingen was used here. Within the Sf nucleus homologous recombination takes place (Luckow, 1991) thus restoring viability and rescuing the virus along with the desired recombinant gene. Due to the large and flexible size of the BV genome (from 80 to 200 kbp) recombinant BV DNA can easily package fragments of foreign DNA up to 15kb in size. With the use of multiple promoter transfer vectors the simultaneous
expression of multiple genes is also possible. Vectors are available in three reading frames, with single or multiple tags for purification and identification (for example 6xHis, GST, and fluorescent proteins such as GFP, BFP and YFP) or with a signal peptide for protein secretion.

Insect cells are so susceptible to BV infection that some labs simply infect larval stage insects that are then ground and extracted for the desired protein. A baculovirus based *Bombyx mori* nuclear polyhedrosis virus-silkworm expression system has also been used successfully to produce the merozoite protein MSP-142 (Pang et al., 2002) and the *P. berghei* ookinete surface antigen as a transmission blocking vaccine antigen (Matsuoka et al., 1996). Cloned cell lines however, remain the most practical choice in a laboratory environment. The most frequently used are *Spodoptera frugiperda* 9 and 21 (Sf9 and Sf21), these are functionally equivalent cell lines that were established from similar larval ovarian tissues. These cell lines grow equally well in insect culture media with or without serum supplement, though, if necessary, it is recommended to practice a gradual progression from serum containing to serum free medium.

In conclusion it may be fair to say that the bacterial method of protein expression of malaria proteins continues largely due to the high degree of standardization inherent to this much used method and to its acceptance by regulatory agencies for the production of proteins for human use. In spite of the difficulties in obtaining active and correctly folded protein and the perils of pyrogen contamination in proteins for use in humans it will probably remain the method of choice in the near future. *Saccharomyces cerevisiae* (Miles et al., 2002) and *Pichia pastoris* will always remain a good alternative choice if correct folding is necessary, however, the list of antigens expressed using BEVS is expanding, from AMA-1 (Anders et al., 1998) to the more recent AMA-142 (Pang, et al., 2002) and the *P. knowlesi* α-protein (Singh et al., 2002).

Considering that one formulation of the promising vaccine candidate, the PfCS antigen adsorbed onto aluminium phosphate, in phase 1 trials is baculovirus produced (Herrington et al., 1992), it is safe to affirm that the logic of preferring this useful expression system is coming to a head.

1.6 Summary of Thesis: Studies on Recombinant PvRII, the Binding Domain of *P. vivax* Duffy Binding Protein

It has been demonstrated that the N’ terminal cysteine-rich region II is the specific domain responsible for mediating erythrocyte binding in the *Plasmodium vivax* Duffy-binding protein (Chitnis and Miller, 1994, Sim et al., 1994 and Ranjan and Chitnis, 1999). Other families of *Plasmodium* erythrocyte binding proteins contain homologous functional domains and are
referred to as Duffy-binding Like (DBL) domains after the first domain identified in *P. vivax*. The family of proteins includes the *P. vivax* and *P. knowlesi* Duffy-binding proteins, *P. knowlesi* β and γ proteins that mediate Duffy-independent invasion pathways (Adams *et al.*, 1990 and 1992), and in *P. falciparum* EBA-175 that mediates invasion by sialic acid residues on glycophorin A (Adams *et al.*, 1992, and Sim *et al.*, 1990) and its homologues EBL1, EBL181, EBL140 which mediate alternative invasion pathways (Peterson and Wellems, 2000). These proteins have been expressed using various expression systems and many have been found to reproduce the expected binding phenotype in specific binding assays (Camus and Hadley, 1985 and Haynes *et al.*, 1988). Antibodies raised against EBPs prevent binding of erythrocytes to mammalian cells expressing region II on the surface and also block invasion of human red blood cells by *P. falciparum* (Pandey *et al.*, 2001) and of rhesus monkey red blood cells by *P. knowlesi* (Singh *et al.*, 2002). Moreover, antibodies to baculovirus expressed region II of EBA-175 inhibited *P. falciparum* growth 50% *in vitro* (Narum *et al.*, 2000).

In this study we have expressed the binding domain, region II, of the *Plasmodium vivax* Duffy-binding protein in the baculovirus expression vector system as a secreted protein in insect cells. We demonstrate that this recombinant PvRII purified from insect cell culture supernatant is active in specific erythrocyte binding assays and that it is highly immunogenic, eliciting high titer antibodies in mice. Mouse antibodies raised against this recombinant PvRII were tested for their ability to recognise *E. coli* produced recombinant PvRII and to inhibit binding of erythrocytes to PvRII expressed on the surface of mammalian cells. These data demonstrate that the baculovirus expression vector system is a viable alternative for the production of functional vaccine components and support the development of PvRII as a vaccine candidate for *P. vivax* malaria.