

Chapter II

Literature Review

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The malaria parasite has been the follower and foe during the history of *Homo sapiens*. The prehistoric man was infected and we are still at risk, despite all efforts to eradicate it during last 100 years. In 1998, the centenary for Ronald Ross discovery of malaria cycle in female Anopheline mosquito was celebrated. This great discovery allowed a line of attack on the disease other than using quinine and other drugs, hoping to eliminate the disease from the world. But, after 100 years of discovery with the huge stride of human scientific progress still 40% of the world populations are under risk. The death accounts for 3-5 million each year and majority are children below five years, infected mainly by *P. falciparum*. The emergence of drug resistance parasites and insecticide resistance mosquito vectors handicapped the global eradication and even containment of malaria. These factors emphasize that there is an urgent need for the development of new effective drugs and vaccine against malaria.

One strategy that could prove very effective, but will take some time to come to fruition is a malaria vaccine (Good *et al.*, 1998). The vaccine must be cheap, safe, effective, and easy to administer. The slow progress towards developing such a vaccine against malaria indicates the magnitude of the task ahead. Attempts to develop a malaria vaccine began early in the twentieth century but in spite of enormous development of biomedical research, especially the immunology and vaccino-biology with reference to malaria, no effective vaccine is available against this disease (Richie and Saul, 2002). The main obstacle of vaccine development is that *Plasmodium* species have evolved multiple mechanism of immune evasion including stage specific antigen expression,

antigenic polymorphism and ability of parasite to manipulate the host immune responses (Brown and Brown, 1965; Anders, 1986; Day and Marsh, 1991; Miller *et al.*, 1994; Brost *et al.*, 1995; Schofield and Tachado., 1996; Patino *et al.*, 1997). Therefore, a vaccine against single stage in the parasite life cycle may not be effective because parasites, which progress to the next stages, may express a different set of antigens. The other very important draw back is that most of the vaccine candidates identified are polymorphic in field populations (Roger and Hoffman, 1998). In spite of the above hurdles, few important observation keep the hopes of malaria vaccine development alive, such as individual continually exposed to infection develops immunity to the disease, passive transfer of antibodies has a dramatic effect for clearing blood stages of the parasite (Cohen *et al.*, 1961) and immunization of radiation attenuated sporozoites induces complete sterile immunity mice, non-human primates and human volunteers (Nussenzweig *et al.*, 1967, Clyde *et al.*, 1973; Rieckmann *et al.*, 1974; Gwadz *et al.*, 1979).

An enormous amount of work is being done in an effort to develop vaccines, which has been reviewed by Cox (1991), Howard & Pasloske (1993), Miller & Hoffman (1998), Engers & Godal (1998) and Phillips (2001). Four species of malaria infect human, which raises initial questions how many vaccines are needed (Richie and Saul, 2002). But at present, *P. falciparum* parasite deserves all attentions because of the variety and severity of the disease it causes (Chen *et al.*, 2002). As mentioned earlier an anti-infection vaccine targeted against a) sporozoites and liver stages of the parasite to protect people from infection, b) an anti-disease/ anti-mortality vaccine targeted against blood stages of the parasites to reduce the mortality and morbidity and c) transmission blocking

vaccine targeting the mosquito stages of parasite for preventing the spread of the disease, are required.

When an infective mosquito bites a human host, sporozoite stage of malaria parasite is injected in to the human body, which makes its way to liver cells with in 30 minutes. It grows and multiplies inside the liver cells (pre-erythrocytic cycle) (Fig 1. 2). Development of vaccine was first realized against sporozoite stages of malarial parasites. Exposure of human volunteers to approximately 1000 to 1,600 irradiated *Plasmodium falciparum*-infected mosquitoes elicited immunity for 3-12 month periods (Herrington *et al.*, 1991; Egan *et al.*, 1993). In addition to it, volunteers immunized by exposure to the bites of mosquitoes infected with *P. falciparum* of Asian origin were protected against challenge by African or South American strain of *P. falciparum* (Clyde *et al.*, 1973). This cross-protection is noteworthy in the light of the polymorphism of sporozoite antigens, which have been shown as a target of vaccine antigens (Lockyer *et al.*, 1989; Robson *et al.*, 1998). The immune mechanism underlying the protection is still unknown (Richie and Saul, 2002). Available evidence indicates that importance of T-cell response on the parasite protein expressed on surface of the hepatocyte may be playing role in protection (Hoffman *et al.*, 1989). The circumsporozoite protein (CSP) has been studied well for its vaccine potential and was the first pre-erthrocytic antigen to be identified and characterized (Yoshida *et al.*, 1980). CSP has an immunodominant epitope of tandem repeats of amino acids and is basically similar in all *Plasmodium* species (Nussenzweig and Nussenzweig, 1985; Hollingdale *et al.*, 1990). Recently two other liver stage parasite antigens namely sporozoite surface protein 2 (SSP2) and the liver stage antigen (LSA-1)

have been characterized. SSP 2 contains an immunogenic region of repetitive sequence and a conserved region involved in adhesion to the host cell (Hedstrom *et al.*, 1990). Immunization of mice with *P. yoelii* -SSP2 protected mice completely against infection (Rogers *et al.*, 1992; Khusmith *et al.*, 1994). The *P. falciparum* homologue of SSP was identified as the Thrombospondin Related Anonymous protein (TRAP) (Robson *et al.*, 1998). Like CSP, SSP2/TRAP contains various repeat regions and varies in sequence and size in different *Plasmodium* species. A recent study indicates that the protein is essential for sporozoite invasions of mosquito salivary glands and mouse hepatocytes (Sultan *et al.*, 1997). LSA-1 is a 200-kDa protein expressed within the parasitophorous vacuole throughout liver stage schizogony and has been found conserved in both repetitive and non-repetitive regions between two strains of *P. falciparum* (Guerin-Marchand *et al.*, 1987; Fidock *et al.*, 1994). Antibodies to the repeat sequences of *P. falciparum* LSA 1 cross reacts with a 230 kDa antigen of *P. berghei* liver stage antigen termed as LSA-2 (Hollingdale, 1990), and mice immunized with LSA 2 peptide were protected against challenge by the *P. berghei* infective bites of mosquitoes. Pfs 16 is 16 kDa protein expressed on the surface of the sporozoites, hepatic stage schizonts and gametocytes of *P. falciparum* (Bruce *et al.*, 1994; Moelans *et al.*, 1995). Antibodies to Pfs 16 inhibit sporozoite invasion of human hepatoma cells and primary human hepatocytes (Moelans *et al.*, 1995). The first synthetic peptide malaria vaccine based on CSP repeat region (NANP)₃ – tetanus toxoid (TT) protein conjugates was subjected to phase I and phase II clinical trials in human volunteers (Herrington *et al.*, 1987; Etlinger *et al.*, 1988). Although the antibody titer elicited was sub-optimal, one of the three vaccinated volunteers was totally protected while the other two had prolonged prepatent periods

following exposure to *P. falciparum*-infected mosquitoes (Herrington *et al.*, 1987). Other than a few proteins, little is known about the antigenic repertoire of pre-erythrocytic stages of malaria parasites. Despite the difficulties in studying the pre-erythrocytic cycle stage, there is sufficient evidence that liver stage antigens will play a crucial role in the development of an effective anti-infective vaccine or a cocktail vaccine against malaria.

The liver schizonts burst and the release waves of merozoites, which enter in to a new environment "Erythrocytes", the most precious tissue of living organisms. The parasite grow and multiplies inside and this cycle is known as erythrocytic cycle, which is the epicenter of pathogenesis of the malaria. Very interestingly, *P. falciparum* parasites stay just for a few days in liver, which is about 3 kg, but in contrast stay for years in red blood cells. The drug susceptibility of the liver stages and blood stages of the parasites are different and also differ in their gene expression and antigenic repertoire. The studies on blood stage of malarial parasites are plenty because of its importance in disease manifestation, availability of *in-vitro* culture of *P. falciparum* malaria parasites. The direct evidence of acquired immunity to blood stages of malarial parasites was available from studies of induced malaria used as a treatment for neurosyphilis earlier in the past century. In these studies, infection with either *P. falciparum* or *P. vivax* led to the species specific immunity, which moderated or prevented subsequent infections, most effectively with the homologous strain but to even to some extent heterologous strains (Yorke and Macife, 1924; Boyd and Matthews, 1939; Covell and Nichole, 1951; Jefferey, 1966). The epidemiological studies have suggested that there are at least two types of naturally acquired immunities that play a role in endemic areas. An anti-disease immunity, which

is rapidly acquired when the children are about 5 years reducing the clinical severity of the disease and the other one is anti-parasite immunity, which develops more gradually, when exposure to multiple strains during consecutive infection. However, the alternative hypothesis that the critical antigens involved in protection are relatively invariant but poorly immunogenic has not been excluded (Rogers and Hoffman, 1998).

One of the front-runners of the blood stage vaccine candidate is merozoites surface protein 1 (MSP-1), which was initially described with a protective monoclonal antibody to *P. yoelii* (Holder and Freeman, 1981). Homologous proteins were identified in *P. falciparum* and other species with molecular masses between 190 and 230 kDa and their potential as vaccine candidates has been investigated (Diggs, Ballou and Miller, 1993). MSP-1, synthesized by the intracellular schizonts is thought to play a crucial role in the process of invading the red blood stages (Blackman *et al.*, 1990). The C-terminal fragment of 19 kDa fragment of MSP-1 is highly conserved throughout the *Plasmodium* species (Del Portillo *et al.*, 1991). Immunization experiment with mice and Aotus monkey with recombinant protein from MSP-1₁₉ of *P. yoelii* and *P. falciparum* were successful in providing protection (Kumar *et al.*, 1995). Another 45-kDa merozoite surface protein (MSP-2) contained a variable central repeat region and highly conserved N- and C-terminal regions. Immunization with synthetic peptides from these conserved regions of *P. falciparum*. MSP-2 protein provided partial protection against *P. chabaudi* challenge in mice (Saul *et al.*, 1992). A cross-sectional survey suggested an association between the presence of antibodies to the conserved regions of MSP-2 and the reduced frequency of clinical malaria episode. MSP-3, a 48-kDa protein identified using purified

IgG, was found to confer partial protection in human passive transfer experiments and the protective epitope is relatively well conserved (Oeuvary *et al.*, 1994). Apical membrane antigen-1 (AMA-1), one of the protein found in rhostry organelles, involved in the invasion of red blood cells was identified as vaccine candidate from the studies with monkey parasite *P. knowlesi* (Deans *et al.*, 1988). AMA-1 is highly conserved in all species of *Plasmodium* and has undergone human trials as a vaccine candidate (Water *et al.*, 1990). RESA, a ring infected erythrocyte surface antigen of human malaria parasite of molecular mass of 155 kDa of *P. falciparum* showed protective role in immunization trials in Aotus monkey against *P. falciparum* infection (Collins *et al.*, 1986). Serine-rich antigen (SERA) is a soluble protein synthesized by late erythrocytic stages of the parasites and secreted in to the parasitophorous vacuole (Delplace *et al.*, 1987) and found to be conserved in different *P. falciparum* strains has shown potential as a vaccine candidate in monkey model (Inselburg *et al.*, 1991; Fox *et al.*, 1997). Immunization experiments with non-polymorphic *P. falciparum* rhostry antigen (RAP-1) protects Saimiri monkey from lethal challenge of *P. falciparum* infections (Ridley *et al.*, 1990). Erythrocyte binding antigen 175 (EBA-175) is a high molecular weight protein localized in the microneme of the parasite and has a role in binding to the erythrocyte receptor. *In-vitro* erythrocyte biding assay has identified the region II as the critical binding domain (Chitinis and Miller, 1994) and antibodies raised against the protein inhibit the growth of *P. falciparum in-vitro* (Daugherty *et al.*, 1997). The most outstanding antigen for vaccine such as PfEMP1 (*P. falciparum* erythrocyte membrane protein 1), which shows considerable diversity but is a potential vaccine candidate for *P. falciparum* by focusing on conserved domain of the protein. A single parasite clone contains roughly 50 different

copies of the gene for variable surface antigen PfEMP1. During a chronic infection, each successive wave of parasitemia expresses a new variant surface antigen, thus allowing parasite multiplication and the presence of antibodies directed to the preceding wave (Saul, 1999). The antigenic polymorphism is a strategy used by the parasites to evade host immune system. In this context, identification of conserved protein or conserved domain may be helpful to overcome these hurdles (Gamain *et al.*, 2001). It is evident that, the conserved gene or conserved region of most of the potential vaccine candidate molecules are protective and will have a wider application for vaccine development. Usually, conserved genes having vital functions in the parasite and are also less prone to mutation and hence can be molecules for vaccine and drug target.

The other important targets are the various protein molecules expressed in sexual stages of malarial parasites and stages inside the mosquito host to block the transmission of infection. Pfs 25 is the first malaria sexual stage antigen gene to be cloned and well studied for its vaccine potentials (Kaslow, 1997). Pfs 25 has shown to induce transmission blocking antibody in mice and monkeys (Barr *et al.*, 1991; Kaslow and Shiloach, 1994) and recently under human vaccine trial. The other promising transmission blocking candidate is Chitinase, which is secreted by Ookinete stages of the malarial parasite to traverse the chitin containing peritrophic matrix (PM) to form sporozoite producing oocysts on the hemocoel side of the midgut (Vinetz *et al.*, 1999; Dessens *et al.*, 2001). Monoclonal antibodies raised against chitinase are shown to block transmission of *P. falciparum*, *P. gallinaceum* and *P. berghei* infection. Earlier, it was also reported that a chitinase inhibitor blocked both parasite infectivity and

transmission (Shahabuddin *et al.*, 1993). The developments of anti-mosquito antigen vaccine, which may block a wide range of vector-borne diseases, are ongoing in a few laboratories.

The only malarial vaccine to undergo extensive field trials in Latin America, Africa, and Southeast Asia is SPf66, a synthetic peptide cocktail developed by Pottarroyo and colleagues (Pattarroyo *et al.*, 1988; M'Coronell *et al.*, 1993). In these field trials, the vaccine, under conditions of high as well as low malaria transmission, gave partial protection in some studies (Valero *et al.*, 1993) and none in others (D'Alessandro *et al.*, 1995). The efficacy of SPf 66 malaria vaccine, concluded on epidemiological data of various trials, was about 23% (Riley, 1995). There is good evidence that malaria vaccine should be multistage and multivalent (Enger and Godal, 1998). As the parasites express different stage specific proteins, vaccine would be effective if it contains antigens from more than one stage. The pox-vectored, multiantigen, multistage vaccine against *P. falciparum*, NYVAC-Pf7 (which includes the transmission-blocking Pfs 25; the pre-erythrocytic antigens CSP, SSP2/TRAP, and the liver-stage antigen 1; and the asexual blood-stage antigens MSP-1, AMA-1 and SERA) underwent phase I/II human trial (Tine *et al.*, 1996, Hill *et al.*, 1998, Ockenhouse *et al.*, 1998). Only one volunteer was completely protected (out of 35 subjects) and others showed delay in time to parasite patency.

The other vital arm of malaria control is chemotherapy. Antimalarial drugs saved millions of deaths in the 20th century. In areas, where malaria transmission is intense,

chemotherapy is the most practical approach for control (Quaye and Sibley, 2002). Identification of new drug targets and drugs is most important because of the quick development of drug resistance in malarial parasites. The three main categories of antimalarial drugs are; (1) 4-amino-quinolines and amino alcohols: Chloroquine and quinine (2) sesquiterpenes: artemisin and derivatives and (3) the antifolates: pyrimethamine, cycloguanil, sulfonamides and sulfones (Trouiller and Olliaro, 1998). *Plasmodium falciparum*, the killer parasite populations are widely resistant to quinolines and antifolates in most of the malaria endemic areas of the world (Phillips, 2001). Recent reports show there is emerging drug resistance to artemisin (Das *et al.*, 2000), which is an important concern for future line of treatment to this deadliest disease. Chloroquine was a chance finding and used for several decades before significant clinical resistance was observed (White, 1998). The chloroquine resistance was reported in 1959 in Thai Cambodia border and in the current situation chloroquine resistance is widely spread. Despite many years of study, the precise mode of action and resistance to quionloine antimalarial drug are still not understood (Macreadie *et al.*, 2000). The polymorphism of *cg2* gene confers resistance in *P. falciparum* (Su, X *et al.*, 1997). Recently, involvement of *Pgh1* (P-glycoprotein homologue) gene as a modulator of resistance to quinolines and other anti-malarial drug was reported (Reed *et al.*, 2000). The chloroquine resistant parasite infected individuals were treated with anti-folate drug successfully and in many endemic countries chloroquine is replaced by anti-folate drugs. In *P. falciparum*, a point mutation in *DHFR* (dihydrofolate reductase) and *DHFS* (dihydropteoate synthase) genes is the major cause of less effectiveness of antifolate drugs. Combination of *DHFR* and *DHFS* inhibitors act synergistically, when either component is compromised, the

effectiveness is dramatically reduced. A point mutation changing Ser 108 to Asn108 in the DHFR gene of *P. falciparum* increases resistance to pyremithamine about 100 fold (Cowman *et al.*, 1988; Peterson *et al.*, 1988). Other point mutations at 51, 59, and 164 also confer resistance to antifolate drug (Sirawaraporn, *et al.*, 1997). Point mutation in DHFS gene is also a predominant mode of resistance to antifolate drugs (Trigalia, *et al.*, 1997; Wang *et al.*, 1997).

In recent days much emphasis is given on the identification of antimalarial target based on molecular mechanism. Enzymes of the *de novo* pyrimidine biosynthetic pathway of *P. falciparum* are focused for antimetabolic therapy. The other important focus on anti-malarial drug target is transport and trafficking protein of malarial parasites in the erythrocytes. Malaria parasite performs the most extraordinary gymnastics of transport and trafficking. In the recent discovery of utilization of host enzyme δ -aminolevulinate dehydratase (ALAD) by the parasite for heme synthesis has added a new twist in parasite biology and chemotherapeutic targets (Bonday *et al.*, 2000). Very little is known about the transport mechanism of including nucleo-cytoplasmic trafficking mechanism of malarial parasites. The recent opinion in MAM 2000 (Molecular Approaches to Malaria in Lorne, Victoria, 2-5 February 2000), suggested that studies in the transport and trafficking mechanism in malaria infected erythrocyte will pay the dividends in terms of identification of new chemotherapeutic targets and development of new and much needed strategies (Dorren *et al.*, 2000).

The prediction of 3- dimensional structure of important functional protein of malaria parasite and development of drug(s) based on it will lead to identification of much needed novel drug against malaria.