CHAPTER I

INTRODUCTION AND REVIEW OF LITERATURE
INTRODUCTION

Malaria is a mosquito transmitted blood infection caused by a protozoan parasite of the genus *Plasmodium*. Since the discoveries of its causative agent, the malaria parasite, in the blood of a soldier by Laveran in 1880, its vector the female *Anopheles* mosquito by Ross in 1897 and by Grassi in 1898 and with the better recognition of the values of Cinchona bark and quinine for the treatment of the disease by Pellitier and Caventou in 1820, much has been known about the life cycle of the parasite, bionomics of its vector, better understanding of the clinical aspects of the disease, its treatment and control strategies during the course of present century (Wernsdorfer and McGregor, 1988, Haworth, 1991).

Despite the intensive investigations and major eradication programmes of WHO (Najera, 1989) malaria still remains one of the major world health problems, particularly in the tropical and subtropical countries, causing profound morbidity and mortality and its a great challenge to the scientists all over the world (Cook, 1989, Miller, 1992). According to the latest WHO report, more than 2000 million people i.e. approximately half of the world population in some 100 tropical and subtropical countries of the world are exposed to malaria of these 500 million live in tropical Africa, the highest endemic area in South of Sahara. According to the statistics of the United Nation’s population division in 1990, malaria is the only disease today apart from AIDS that shows a significant rising tendency. Every year 110 million clinical cases of malaria occur throughout the world, of which 80-90% are Africans. 1-2 million people mostly children under 5 years of age die of malaria each year, again the maximum mortality occurs in tropical Africa. Apart from Africa 95% of 5.2 million cases are reported from other 25 tropical and subtropical countries of which mostly from India (39%), Brazil (11%) and rest from other countries like, in descending order, from Afghanistan, China, Burma, Sri Lanka, Thailand, Vietnam etc. (Liisberg, 1991, WHO, 1991, World Malaria Situation, 1992). As far as India is concerned, according to the WHO estimation in 1990, out of the total Indian population, more than 95% are at risk due to malaria, a total of 1777253 clinical cases of malaria were reported in India with a wide variation observed from states to states (Dutta and Bhalwar, 1991, Kondrashin, 1992). In some countries the gravity of malaria problem is becoming so much staggering that not only the health of the population is threatened but also the social and economical progress of the communities (Shepard, 1991).
Malaria is thus one of the most serious and widespread tropical diseases in the world today and a great threat to mankind.

The malaria parasite is a haematozoan, i.e. the protozoan that lives in the blood and it is the small *Anopheles* mosquito which transmits the parasite by bitting, at random first a malaria patient and then a healthy person. Four species of *Plasmodium* infecting mankind are *Plasmodium falciparum*, *P. vivax*, *P. ovale* and *P. malariae* of which *P. falciparum* is the most dreaded species of human plasmodia. Apart from human, so many other species of the *Plasmodium* have been identified infecting monkeys (*P. brasilianum*, *P. coatneyi*, *P. cynomolgi*, *P. fieldi*, *P. fragile*, *P. gonderi*, *P. inui*, *P. knowlesi*, *P. simium* etc.), rodents (*P. atheruri*, *P. berghei*, *P. chabaudi*, *P. vinckei*, *P. yoelii*) and avians (*P. cathemerium*, *P. gallinaceum*, *P. lophurae*, *P. relictum*).

Fig. 1 is showing the diagrammatic representation of the life cycle of a primate (human) malaria parasite. Malaria parasite undergoes a complex multistage developmental cycle which alternates between a vertebrate host (man) and an invertebrate vector host (the female *Anopheles* mosquito). The life cycle begins when the sporozoites (the infective stage) from the mosquito's salivary gland are injected into the blood stream during the bite of an infected female *Anopheles* mosquito. With in an hour the sporozoites disappear from the blood circulation and specifically attach and invade the liver parenchymal cells. Within the liver cells the sporozoites mature and by asexual nuclear division produce multinucleate schizonts (Exoerythrocytic schizogony). At this hepatic or exoerythrocytic stage, the infection is asymptomatic and occur over a period ranging from days to months (average 2 to 4 weeks) depending on the species of *Plasmodium*. The multinucleate schizonts along with the infected liver cells, eventually burst releasing thousands of individual merozoites (upto 30000 per sporozoite depending on the species of *Plasmodium*). These merozoites enter into blood stream and are able to specifically attach and invade the erythrocytes and this initiates the erythrocytic phase of the life cycle. Within the erythrocytes, the merozoites subsequently develop and differentiate into rings, trophozoites and finally by asexual nuclear division get converted into multinucleate (8 to 24 daughter nuclei depending on species of *Plasmodium*) schizonts (erythrocytic schizogony). The parasitic cytoplasm also devides and new merozoite membrane then forms at the segmenter stage. Rupture of the segmenter infected erythrocytes releases another generation of extracellular merozoites that rapidly reinvade new erythrocytes.
FIG. 1: Schematic drawing of the life cycle of a primate malaria parasite showing the stages (I to VII) that might act as targets for vaccine induced immunity. (Legends)
*Legends for fig. 1:

E-E: Exo-erythrocytic. RBC: Red blood cells. PRBC: Parasitised red blood cells. Numbers (I to VII) refer to the various stages at which the host immune responses are believe to act against the parasite and form the conceptual basis for development of diverse malaria vaccine strategies:

(I) Sporozoite (Infection Blocking) Vaccine: The injected sporozoites in the blood are susceptible to the antibody mediated immunity (AbMI) as well as cell mediated immunity (CMI).

(II) Liver stage (Exo-erythrocytic) Vaccine: The early developmental stages in liver are target for CMI.

(III) Merozoite (Invasion blocking) Vaccine: The invading merozoites are accessible to AbMI as well as CMI.

(IV) Blood stage (Erythrocytic) Vaccine: The parasitized RBC and intraerythrocytic asexual stages are prone to AbMI as well as CMI.

(V) Antitoxic (Antidisease) Vaccine: The toxic substances released by the parasite may be neutralize by inducing specific immunity.

(VI) Sexual stage (Transmission blocking) Vaccine:

(VI-A) Prefertilization stages (Gametocytes) Vaccine: The Gametocytes produced in the blood are prone to AbMI as well as CMI. Abs against Gametes in blood meal inhibit fertilization in mosquito gut and thereby prevent their further development and transmission of malaria.

(VI-B) Post-fertilization stages (Zygote & Ookinete) Vaccine: Attempts have also been made to develop vaccine against Zygote and Ookinete stages.

(VII) Mosquito stages: There is no evidence of any immunity against further developmental stages in mosquito.
and thereby perpetuating the erythrocytic cycle of the parasite growth and so the blood parasitaemia rises. The duration of erythrocytic cycle depends on the species of the *Plasmodium* and is usually 24, 48, 72 hrs. This erythrocytic phase of the parasite is directly responsible for morbidity, its clinical symptoms and mortality due to malaria. During this erythrocytic phase of the parasite, a portion of the blood stage merozoites after invading the RBCs, due to some unknown factors get differentiated into male and female gametocytes i.e. the sexual stages of the parasite. The gametocyte infected erythrocytes do not rupture and when these are taken by the mosquito during the blood feeding, unlike uninfected erythrocytes and other asexual stages infected erythrocytes, they are not digested in the mosquito gut rather they undergo gametogenesis producing male (micro) and female (macro) gametes. In the midgut of the mosquito, the male microgamete undergoes exflagellation producing 6-8 microgametes. The microgametes fertilize the large female gametes and the resultant zygotes elongate and rapidly differentiate into motile ookinetes. The ookinetes pierce the gut wall and eventually come to rest (extracellularly) between the basement cell membrane and the basal laminae of the midgut wall of the mosquito and round up forming the oocysts. The oocysts grow and usually within 7 to 15 days (mainly depends on the environmental conditions, particularly those of temperature and humidity), by asexual nuclear division (sporogony) produce several thousands sporozoites. The number of sporozoites produced within each oocyst depends on the species of *Plasmodium* (Rosenberg and Rungsiwongse, 1991). A single mosquito may carry several hundred oocysts on its midgut wall. The sporozoites are released from the oocyst and via the haemocoel finally lodge into the lumen of the salivary gland of the mosquito where they remain viable for an indefinite period until injected into another vertebrate host during the frequent blood meals. According to the latest view of the exoerythrocytic schizogony, in some species of plasmodia (*P. vivax, P. ovale, P. cynomolgi*), a portion of the sporozoites after entering the liver parenchymal cells get differentiated into a dormant hypnozoite stage which remain viable for a longer period and eventually grow into schizont and are responsible for relapses (Cogswell, 1992).

The morbidity, its pathology, clinical symptoms and mortality due to malaria is mainly caused by the asexual blood stages of the parasite. The clinical symptoms are characterized by paroxysms of periodic high fever with feeling of chills and vigour accompanied by headache, muscular pain, gastrointestinal upsets which then followed by profound sweating and patient
then return to normal. The fever is associated with the bursting of schizont infected erythrocytes and the severity of the disease in usually proportional to the number of parasitized red cells. The intense destruction of the red cells may lead to other pathological symptoms and may cause anaemia, anoxia, haemoglobinuria, hypoglycaemia, jaundice and neuropsychiatric symptoms. Other pathological symptoms include enlargement of spleen and liver. In case of severe \textit{P. falciparum} malaria, the microvessels of the heart, lungs, kidneys, small intestine, liver, brain and eye may get blocked due to sequestration of parasitized erythrocytes and thus preventing the normal flow of the blood and may cause haemorrhage and anaemia. Sequestered cells that clog the brain capillaries may reduce blood flow sufficiently and it may cause confusion, lethargy and unarousable coma and lead to cerebral malaria. The infection by \textit{P. falciparum} is called falciparum malaria or malignant tertian malaria. \textit{P. vivax} is the cause of vivax malaria and is called tertian fever or benign tertian malaria. The malaria caused by \textit{P. ovale} is called ovale malaria which has close similarities to vivax malaria. The infection of \textit{P. malariae} (malariae malaria) results in the quartan malaria fever. Species responsible for the highest mortality rate especially among children is \textit{P. falciparum}.

The biological and morphological similarities between human and nonhuman primate malarias make the later good animal model of human malarias providing suitable laboratory host available. Much of our knowledge of human malarias have come from the studies on animal malarias in laboratory animals. \textit{P. knowlesi} (24 hr blood cycle), \textit{P. coatneyei}, \textit{P. fragile} (48 hr blood cycle) are extensively used as a model for \textit{P. falciparum} (48 hr blood cycle) type malaria. \textit{P. cynomolgi}, \textit{P. gonderi} (48 hr blood cycle) are excellent model for the study of \textit{P. vivax} (48 hr blood cycle) type malaria. \textit{P. fieldi}, \textit{P. simium} (48 hr blood cycle) used as a model for \textit{P. ovale} (48 hr blood cycle). \textit{P. inui}, \textit{P. brasiliannum} (72 hr blood cycle) used as a biologic model for \textit{P. malariae} (72 hr blood cycle). In all these cases rhesus monkeys (Macaca mulatta) and in some cases (\textit{P. fieldi}, \textit{P. simium}, \textit{P. brasiliannum}), Aotus or Saimiri monkeys are used as laboratory host.

In 1956, prompted with the advent of the residual insecticide dichlorodiphenyl-trichloroethane (DDT) and antimalarial drug chloroquine, WHO introduced the multibillion dollar Global malaria eradication programme based on vector control and chemotherapy (Najera, 1989), but the initial overwhelming success of effective control were turned up in
a great setback due to the widespread resurgence of malaria by the mid-1970s and this was attributed to the development of drug resistance in *Plasmodium* and insecticide resistance in the mosquitoes (Davidson, 1982, Werndorfer, 1991). The chloroquine and multiple drug resistant strains of *P. falciparum*, the most malignant species of human malaria, are spreading in most of the tropical countries (DasKhatri, 1991, Rastogi, 1991, Louis et al., 1992, Wongsrichanalai, 1992) and now *P. vivax* is also gaining resistance to the drug (Collignon, 1992, Murphy et al., 1993). This grim situation led the scientists and research organisations, such as WHO, to the revival of their approaches in malaria research not only for finding the better chemotherapeutic measures, but also towards finding an immunological solution to the disease i.e. the possibilities of producing an effective malarial vaccine as well as better immunodiagnostic test (WHO, 1988a,b, 1993, Howard, 1989, Marshall, 1990, Cherfas, 1990a, Dhar, 1993).

During malaria infection, the parasite remain susceptible to both antibody mediated as well as cell mediated immune defence mechanism of the host. In the recent years, there have been continuous efforts for developing subunit humoral response inducing malaria vaccine as well as malaria vaccine strategy based on T-cell and the induction of cell mediated immunity. In fig. 1 the number I to VII refer to the stages at which the parasite destructive host immune responses are believed to act and form the conceptual basis for development of diverse malaria vaccine strategies.

The blood stages of malaria parasite are promising target not only for developing the new antimalarial drugs but also for the development of antimalarial vaccines and immunodiagnostic tests, because these stages are directly responsible for the morbidity, its clinical symptoms, pathology and mortality due to malaria. Accordingly a survey of literature was done on immunochemical aspects of malaria with special reference to the studies related to the immunodiagnosis and vaccine development against asexual erythrocytic stages of malaria parasites.
REVIEW OF LITERATURE

ISOLATION AND PURIFICATION OF BLOOD STAGES OF MALARIA PARASITES

For conducting the studies on immunochemical, biochemical, molecular and other aspects of malaria, the first and most important thing is to get the malaria parasites in the most purified form at a desired particular stage. The source for getting the asexual blood stages of Plasmodium may be the blood obtained either from the malaria patients, from infected experimental animal hosts or from the in vitro culture of the parasites. The presence of various unwanted blood components such as platelets and leucocytes and erythrocyte membrane components may interfere the study and ultimately lead to the misinterpretation of the data (Howard et al., 1978). It has also been reported that the quantity of leucocytes in the blood circulation generally increases above the normal value during the malaria infection (Homewood and Neame, 1976). The complex life cycle of the parasite and its intimate association with the host tissues make the isolation of the intraerythrocytic parasite in an intact and purified form, a technically difficult task. Several mechanical, chemical and immunological methods have been used to isolate the malaria parasites and their constituents (Hamburgher and Kreier, 1980).

In the first step of centrifugation and washing of infected blood, the plasma and buffy coat are removed and along with a population of number of platelets and leucocytes are also reduced (Zuckerman et al., 1967a, Beutler, 1971) but some parasitized erythrocytes which lie just below the buffy coat may also be losing while removing the buffy coat (Brown et al., 1968a, McAlister and Gordon, 1976). The further removal of the platelets was achieved by passing the blood through the column of glass beads (O’Brien and Heywood, 1967, Shiebel and Miller, 1969, Goldman et al., 1992). Addition of adenosine diphosphate in the blood causes clumping of the platelets and will facilitate their removal by column (McAlister and Gordon, 1976, Scheibel and Miller, 1969). Various methods and techniques have been employed for the further removal of the leucocytes from the infected blood such as by passing through a column of filter paper or cellulose powder (Shiebel and Miller, 1969, Cook et al., 1969, Baggaley and Atkinson, 1972), by applying sedimentation velocity technique using dextron (Zuckerman et al., 1967a), by gradient centrifugation using sucrose (Williamson and
Cover, 1966), Ficoll hypaque solution (Wallach and Conley, 1977), by centrifugal elutriation technique (McEven et al., 1971, Russman et al., 1982). Brown et al. (1966) eliminated the leucocytes by millipore filtration after releasing the parasite from the infected cells using 5 μM pore size. Among the various methods employed for the separation of leucocytes, the separation on Whatman CF-11 cellulose column has been found most suitable because of its simplicity and efficiency (Richards and Williams, 1973, Homewood and Naeme, 1976, Howard et al., 1978, Waki and Suzuki, 1986, Goldman et al., 1992). The column of sulphaethyl cellulose was also used for the removal of leucocytes (Howard et al., 1978). The cellulose column over a layer of glass beads facilitate the combined removal of platelets and leucocytes (Scheibel and Miller, 1969, McAlister and Gordon, 1976).

Most of the techniques used for concentrating the parasitized erythrocytes (PRBC) from the infected blood are based on the differences in the densities of the PRBC and normal RBC (NRBC) (Williamson and Cover, 1966, Miller and Chein, 1971, Heidrich, 1988). In one of the earliest study, Eaton (1938) allowed the schizont infected rhesus blood to settled down slowly and collected the increased concentration of schizont infected erythrocyte just below the buffy coat. Subsequently density gradient centrifugation techniques were also employed by different workers for the separation of parasitized erythrocytes using various gradient materials such as human albumin (Ferreebee and Geimen, 1946), bovine albumin (Rowley et al., 1967, Eisen, 1977, Siddiqui et al., 1978a), sucrose (Williamson and Cover, 1966), phthalate (Miller and Chien, 1971), Strachtan II (McAlister and Gorden, 1976), physiogel (Trager and Jensen, 1976, Reese et al., 1979), plasmagel (Pasvol et al., 1978), gelatin (Jensen, 1978), Metrizamide, a trisodium benzamide derivatives of glucose (Engui and Allisen, 1979), Ficoll (Eling, 1977, Mrema et al., 1979) and percoll (Saul et al., 1982, Rivadeneira et al., 1983, Andrysiake et al., 1986, Waki and Suzuki, 1986, Ihalamulla and Mendis, 1987, Wunderlich et al., 1987, Haider et al., 1988, Handunnetti et al., 1992).

The intraerythrocytic plasmodia from the parasitized host RBCs can be released by applying various physical and chemical procedures. Some of the physical procedures employed include the hypotonic lysis of the infected erythrocytes (Stein and Desowitz, 1964, Chavin, 1966), by French pressure cell lysis technique (Cook et al., 1969, D'Antonio, 1972), nitrogen cavitation technique (Wallach and Conley, 1977, Nilni et al., 1985) or by gentle sonication of the parasitized erythrocytes (Kreier et al., 1976) or by mechanical shearing such
as by forced extrusion through a syringe fitted with a 27 gauge needle (Chow and Kreier, 1972) or through a filter membrane of 35 μM pore size (McAlister and Gordon, 1977). The chemical procedures have also been employed by different workers for releasing the plasmodia from the infected erythrocytes such as by Ammonium chloride treatment (Martin et al., 1971, Prior et al., 1973), by saponin lysis (Zuckerman et al., 1967a). Subsequently the technique of saponin lysis was used efficiently by number of workers with slight modification in the amount of saponin, incubation time and temperature (Jerusalem and Elign, 1969, VanDyke et al., 1977, Kan and Siddiqui, 1981, Beaumelle et al., 1987, Kirande et al., 1991). The mature stages of the intraerythrocytic plasmodia (Trophozoite or Schizont) may also be released by sorbitol lysis (Hoppe et al., 1992, Handunnetti et al., 1992) but the ring infected and uninfected erythrocytes are not lysed by sorbitol (Lambros and Vanderberg, 1979), so they can be removed by filtration through 80 μM filter after agglutination with either anti-erythrocytic monoclonal antibodies or wheat germ agglutinin. Plasmodia may also be released by immunolysis i.e. by incubating with specific anti-host erythrocytic immune serum and compliment with gentle agitating to enhance the lysis and followed by centrifugation to isolate the free parasites (Trager et al., 1972, 1990), by lectin agglutination and seiving procedure (Nilni et al., 1981, Heidrich et al., 1982). The contamination of erythrocytes and their ghosts as well as the parasites still surrounded by these membranes may be eliminated from the freed parasites by applying different methods such as by differential centrifugation (Trager et al., 1990), by density gradient centrifugation using bovine serum albumin (Eisen, 1977), sucrose (Siddiqui et al., 1978c), picoll or percoll (Nilni et al., 1981, 1985, Wunderlich et al., 1987), and free flow electrophoresis (Heidrich et al., 1982) or by immunoaffinity chromatography (Hoppe et al., 1992).

The immunochemical studies are conducted with the soluble extracted materials of the isolated parasites. For solubilization of parasite preparation, the nonionic detergents such as Berot EMU-043 or Triton-X-100 was found most suitable as it causes minimum denaturation of proteins during solubilization (Bjerrum and Lundahl, 1975, Deans et al., 1978, Kaushal et al., 1983, Gonzalez-ceron and Rodriguez, 1991).

For getting the desired synchronised stage of development, certain procedures are required. The species such as Plasmodium lophurae, P. chabaudi, P. knowlesi cause highly synchronous infections, facilitating the isolation of defined stages of these species relatively
easier by judicious choice of the time of blood collection (Brown et al., 1968a, Trager et al., 1972). In case of rodent malaria parasite *P. berghei*, not naturally synchronised or loose synchrony in culture, in such cases the gradient centrifugation may be applied to get the desired stage of parasites (Shungu and Arnold, 1971, McAlister and Gorden, 1977). The synchronised stage of *P. falciparum* from culture may be obtained by treating the parasitized erythrocyte with 5% sorbitol for 2-5 min at 37°C, this destroyed the mature form of the parasite and only the ring stages survived which kept for further cultivation (Lambrose and Vanderber, 1979). α-difluoromethylornithine (DFMO) was also used to synchronize the trophozoite stage of the *P. falciparum* (Hoppe et al., 1991, 1992).

**IMMUNIZATION AGAINST ASEXUAL BLOOD STAGES OF MALARIA PARASITES**

The induction of protective immunity against erythrocytic stages of plasmodia derived from avian, rodent, simian and human malarias has been shown in appropriate experimental animals by immunizing with extracellular merozoite as well as with parasitized erythrocytes, either irradiated, heat inactivated, attenuated, killed or fractionated (Siddiqui, 1980, Deans and Cohen, 1983).

In some of the earliest attempts of immunization with the avian species of plasmodia, the protection of canaries immunized against heat treated or formalin killed erythrocytic stages of *P. cathemrium* (Gingrich, 1941) and of ducks immunized against killed erythrocytic stages of *P. lophurae* (Jacobs, 1943) have been demonstrated. Subsequently immunization of ducks with *P. lophurae* (Freunds et al., 1945a, Coffins 1951) and with *P. cathemrium* (Thomas et al., 1947) each incorporated with Freund’s complete adjuvant (FCA) has shown and confirmed the effectiveness of the use of adjuvant (FCA) in immunization against *Plasmodium*. Kilejian (1978) for the first time employed purified protein for immunization against malaria. He immunized ducklings with a purified histidine rich protein (HRP) isolated from *P. lophurae* infected erythrocytes and protective immunity was shown to induced even without FCA.

In case of rodent species of plasmodia, the effective immunization against asexual blood stages of *P. berghei* was attempted using irradiated infected blood (Wellde and Sadun, 1967), erythrocyte free parasite (Jerusalem and Eling, 1969, D’Antonio et al., 1970) as well
as cell free extract and soluble homogenate (Desowitz, 1967, Zuckerman et al., 1967b, Saul and Kreier, 1977, Grathaus and Kreier, 1980). Active immunization of mice against saponin lysed formalin fixed *P. yoelii* parasitized cells mixed with *Bardetella pertussis* organisms (Playfair et al., 1977) and Triton extracted erythrocytic stages of *P. yoelii* mixed with saponin (Playfair and Desouza, 1986) has been reported. Kumar et al. (1990) have reported the high level of protection in mice immunized with killed *P. vinckei* antigen with attenuated *Typhemurium*.

Among the simian plasmodia, in some of the earliest attempt of immunization against the blood stages of malaria parasite, although the induction of malaria antibodies in rhesus monkeys (*Macaca mulatta*) against the *P. knowlesi* was demonstrated but the immunity was not protective (Eaton and Coggeshall, 1939, Short and Menon, 1940, Ray et al., 1941). Subsequently the protective immunity against *P. knowlesi* was demonstrated for the first time by immunizing monkeys subcutaneously with formalin treated schizont infected erythrocyte of *P. knowlesi* emulsified in paraffin oil containing killed tubercle bacilli i.e. the freund’s complete adjuvant (Freund’s et al. 1945b, 1948) and subsequently by other workers (Targett and Fulton, 1965, Brown et al., 1970a) and it was also reported that the better immunity was afforded by intramuscular rather than subcutaneous route of immunization (Targett and Fulton, 1965). Subsequently the induction of protective immunity in rhesus monkey was demonstrated by immunizing against the partially purified fraction i.e. PPF (D’Antonio et al., 1971) and various nonviable sucrose density gradient fractions (Simpson et al., 1974). Plasmodial lyophilized antigen has also been used successfully for immunization (Schenkel et al., 1975, Cabrerra et al., 1976). In some studies, the blood stage merozoite was found most potent immunogen with 90-100% survival rate in rhesus monkeys against normally lethal challenge (Mitchell et al., 1977a, Richards et al., 1977). However, the superiority of merozoite antigen could not be established in a comparative evaluation of the efficacy of three nonviable blood stages’ antigens (merozoite, schizont, a schizont’s FPC fraction i.e. a schizont’s fraction obtained by French press cell disruption technique) of *P. knowlesi* (Rieckmann et al., 1978, 1979). Khanna et al. (1991) effectively immunized the rhesus monkeys against erythrocytic stage of *P. knowlesi* and high titres of antimalarial antibodies were recorded.

Immunization attempts were also made with the aim to define specific protective
antigens having potential value for vaccination. A 74 KD glycoprotein isolated from *P. knowlesi* schizont infected rhesus erythrocytes was shown to induce protective immunity against potentially lethal infection of *P. knowlesi* (Schimidt-Ullrich *et al.*, 1986b). In other studies, a 140 KD (Miller *et al.*, 1984, Klotz *et al.*, 1987) and a 66 KD (Deans *et al.*, 1988) antigens of *P. knowlesi* were shown to induce protective immunity in rhesus monkeys.

In some of the earliest attempts of immunization with the human malaria parasite the killed *P. vivax* blood stage antigen was used to vaccinate human volunteers but without any success (Heidelberger *et al.*, 1946). Immunization of owl monkeys with irradiated *P. falciparum* infected erythrocytes however could induce only partial protection (Sadun *et al.*, 1969, Wellde *et al.*, 1979). Siddiqui (1977) for the first time reported the successful immunization of owl monkeys against *P. falciparum* blood stage (60-70% merozoites) antigen emulsified in FCA and demonstrated the 100% protection against strain specific challenge. The work of Siddiqui (1977) has also been independently confirmed by Mitchell *et al.* (1977b). Subsequently Siddiqui *et al.* (1978b) have also demonstrated protection against heterologous strain of *P. falciparum*.

During the recent years immunization attempts have been done with specific blood stage antigens of human malaria (Miller *et al.*, 1986, Moreno and Patarroyo, 1989). Various purified recombinant and synthetic blood stage antigens belonging to merozoite surface (Patarroyo *et al.*, 1988, Hui *et al.*, 1991, Saul *et al.*, 1992, Amador *et al.*, 1992a) infected erythrocyte surface antigens such as Pf 155/RESA i.e. ring infected erythrocyte surface antigen (Collins *et al.*, 1991, Wahlin, 1990). SERA i.e. serine repeat antigens (Barr *et al.*, 1991, Inselberg *et al.*, 1991), HRPA i.e. histidine rich protein antigens (Knapp *et al.*, 1992), glycophorin binding antigens (Aronson *et al.*, 1991), rhoptries antigens (Perrin *et al.*, 1985), were tried for vaccination and various degrees of protection were observed using either the conventional FCA or several other adjuvants. Attempts has also been done to immunize the *Aotus* monkeys passively with human antibodies against Pf 155/RESA antigens (Berzins *et al.*, 1991).

The importance of adjuvant in inducing protective immunity against plasmodium was reported for the first time by Freund’s *et al.* (1945a,b). He employed paraffin oil containing killed tubercle bacilli, latter known as Freund’s Complete Adjuvant (FCA), for immunization against malaria. Subsequently its efficacy was established by other workers (Targett and

Out of the so many adjuvants tried as reviewed above, no other adjuvant could be strongly found more potent and better substitute for FCA. However, in immunization with P. berghei antigen in rats using Saponin and FCA as adjuvants, it has been shown that saponin was better than FCA (Saul and Kreier, 1977). Siddiqui et al. (1978d) reported the successful replacement of FCA by stearoyl-MDP for immunization against malaria. In other comparative studies, the adjuvant formulations based on synthetic B-30-MDP (a lipophilic muramyl dipeptide derivative), LA-15-pH (a synthetic equivalent of monophosphoryl lipid A) and alum was found as effective as FCA in inducing antibody response against gp 195 protein but there were distinct differences in the recognition of B cell epitopes by different adjuvants and it was also demonstrated that the MHC control of antibody specificity can be related to adjuvant used for immunization and thus suggested that the influence of adjuvants should be a major consideration in studies on immunogenic recognition as well as the design of modern subunit vaccines (Hui et al., 1991, Hui and Chang, 1992, Millet et al., 1992). The major problem of adjuvant research has been to dissociate the immune response from inflammatory
side effects. FCA, the widely used potent adjuvant in experimental animals are so fraught with side effects that it was not licenced even for veterinary use (Jones et al., 1989). At present the only adjuvant licensed for human use are mineral gels such as aluminium hydroxide which has been included in malaria recombinant and peptide material vaccine tested in man (Herrinton et al., 1987, Patarroyo et al., 1988, 1992).

**IMMUNOASSAYS FOR MEASURING ANTIBODY AND DETECTING CIRCULATING ANTIGEN**

Several immunoassays viz. Enzyme linked immunosorbent assay, Indirect haemagglutination assays, Immunofluorescence assay have been widely used for detecting the antimalarial antibodies as well as the circulating antigens in the host serum.

**ENZYME LINKED IMMUNOSORBENT ASSAY**

The enzymes conjugated with antigens and antibodies have been used initially by several workers for localization of antigens and antibodies in tissue section (Avrameas, 1970) as well as for quantitative determination of antigens and antibodies in immunoelectrophoresis and gel diffusion (Avrameas, 1969, Stanislawski, 1970). Subsequently, the competitive enzyme-linked immunosorbent assay (ELISA) for the quantitation of antigens or antibodies using enzymes conjugated with antibodies or with antigens fixed on a solid phase, was independently developed by two groups of workers, one in Sweden using alkaline phosphatase enzyme, reported in a series of three papers (Engvall and Perlmann, 1971, 1972, Engvall et al., 1971) and other in Holland using horse radish peroxidase enzyme (Vanweemen and Schurs, 1971). Voller et al. (1974, 1975) for the first time introduced this technique of ELISA in the field of malaria for detecting the antimalarial antibodies during *P. vivax* and *P. falciparum* infection using crude antigenic preparation from *P. falciparum* schizont infected erythrocytes derived from *Aotus* monkey (Voller et al., 1974) and from *P. knowlesi* schizont infected erythrocytes derived from rhesus monkey (Voller et al. 1975). Subsequently this technique of ELISA has been extensively employed in the field of malaria immunology by various groups of workers.

A variety of solid phases were employed in ELISA as a carrier surface for coating antigens, such as agarose beads (Streefkerk and Deelder, 1975), desposable polystyrene tubes (Engvall and Perlman, 1972, Voller et al., 1975) or more suitable and generally
employed disposable polystyrene microtitration plate (Voller et al., 1974, 1977, Spencer et al., 1979a,b, Mahajan et al., 1981, Dutta et al., 1984, Srivastava et al., 1985, Lee and Lambros, 1988). Nitro cellulose membrane has also been employed as carrier surface for coating the antigen in ELISA (Petros et al., 1989). Among the variety of enzymes available, the commonly used enzyme marker in ELISA for labelling the antiglobulin conjugate are alkaline-phosphatase (Voller et al., 1974, 1975, 1976, 1980, Mackey et al., 1982, Knobloch and Hermentis, 1987) and horse radish peroxidase (Spencer et al., 1979a,b, Mahajan et al., 1981, Tharavenij et al., 1982, Ray et al., 1983, Demerts et al., 1987). A number of enzyme substrates (chromogens) have been used in ELISA, such as P-nitrophenyl phosphate (Voller et al., 1974, 1975, 1980), aminosalicylic acid (Voller et al., 1976), ortho-phenyldiamine (Spencer et al., 1979a,b, 1981, Mahajan et al., 1981, Dutta et al., 1984), 4 chloro-L-naphthol (Londner et al., 1987) and 3-3', 5-5'-tetramethylbenzidine (Lee et al., 1990).


A new technique of DOT-ELISA based on the same principle of ELISA for the rapid detection of malarial antibodies and which could be able to detect the malaria antibodies upto
a dilution of 1/64000 in patient sera has been reported (Londner et al., 1987). Some others more sensitive, simple, rapid and easily visible ELISA systems in which reaction can be read with naked eye without using ELISA reader, have been reported for the detection of antimalarial antibodies specially convenient in the field study, such as one, using urease as an enzyme marker and urea as a substrate, named as “ELISA-U” (Lee and Lambros, 1988) and other one using avidin biotin peroxidase complex enzyme i.e. “ABC-ELISA” (Sato et al., 1990). Baruch et al. (1991) employed a sensitive fluorescence cell ELISA method for the detection of antimalarial antibody. Dziegieł et al. (1992) described a novel ‘U’ chain capture ELISA for the detection of IgM antibodies against P. falciparum antigen. Boraker et al. (1992) have reported the use of Acoustic probe-based ELISA. ELISA system has also been employed for the diagnosis of malaria based on detecting circulating antigen using refined antibody (IgG), such as against P. vivax (Wang et al., 1987), P. falciparum (Bualombai et al., 1990). Khullar and Sehgal (1989) employed ELISA for the detection of circulating antigens in the sera of mice infected with P. berghei. Avidor et al. (1992) used the cross reacting monoclonal antibodies against P. berghei for the identification of P. falciparum antigen using a sensitive ELISA system. An improved and more sensitive sandwitch ELISA technique for the detection of blood stage malarial antigen in human plasma by employing monoclonal antibodies against P. falciparum (Dubary et al. 1990) and P. vivax (Gao et al., 1991) has also been reported.

In a comparative evaluation ELISA is found more superior and sensitive than other immunological techniques such as IHA and IFA (Mahajan et al., 1981, Khanna, 1991).

INDIRECT HAEMAGGLUTINATION ASSAY

The idea that the various substances can be absorbed on the surface of red blood corpuscles without significant damage to the cells, and these can then be agglutinated by serum antibodies against the attached group (Pressman et al., 1942, Middlebrook and Dubos, 1948) led the evolution of haemagglutination test to be a valuable technique for detecting circulating antibodies developed against variety of antigens, both polysaccharide and protein in nature (Boyden, 1951, Ingraham, 1952, Wright and Feinberg, 1952, Stavitsky and Arquilla, 1955, 1958, Lunde and Jacobs, 1959). Various coupling agents were used to bind antigens to red blood cells for passive haemagglutination test, such as dizotised arsalinic acid
and bisdiazotized benzidine (BDB) (Pressman et al., 1942), tannic acid (Boyden, 1951), 1,3 difluoro-4,6-dinitrobenzene (Ling 1961), tolylene-2-diisocyanate (Gyenes and Sehan, 1964) and corboiimide (Johnson et al., 1966). The BDB and tannic acid techniques are well established methods for detecting small quantities of antibodies and antigens (Stavitsky, 1964). These methods in general, however, have the disadvantage of being somewhat complicated and time consuming. A more easier and rapid chromic chloride (CrCl₃) method for binding of antigens to red cells is reported (Gold and Fudenberg, 1967). The problem of poor stability and variable sensitivity among different lots of sensitized erythrocytes, prompted the use of some fixative agent to stabilize the red blood cells for their use in haemagglutination test. Out of the various stabilizing agent employed, such as formaldehyde (Daniel et al., 1963), pyruvic aldehyde (Hirata and Brandriss, 1968), the glutaraldehyde fixation of erythrocytes was found superior and the cells were found stable for at least six months (Bing et al., 1967. Hirata and Brandriss (1968) employed the stabilized sheep, chicken and rabbit erythrocytes and reported the optimum conditions such as pH, antigen concentration and the duration of contact with antigen for the better coating of variety of polysaccharide and protein antigen with RBCs.

The technique of haemagglutination has been employed earlier in the serology of parasitic infections, such as schitosomiasis and trichinosis (Kagan, 1955, Kagan and bargai, 1956) and toxoplasmosis (Lunde and Jacobs, 1959). Desowitz and Stain (1962) for the first time introduced this technique in the field of malaria for the quantitation of antibody titres of rat antisera against *P. berghei* antigen using tanned sheep erythrocytes and subsequently in human, Stein and Desowitz (1964) employed formalized tanned sheep erythrocytes sensitized with *P. vivax* for measuring the malaria antibody in patient sera. Later on, the simplicity and rapidity of this test, called as indirect haemagglutination assay (IHA), led the several groups of workers to employ this technique for immunodiagnosis and epidemiological surveillance of human malaria using homologous antigens from *P. falciparum* (Meuwissen, 1974, Meuwissen et al., 1974, Mathews et al., 1975, Gupta et al., 1979, Ray et al., 1981, Dubey et al., 1989, Marwah et al., 1989), *P. vivax* (Mathews et al., 1973, 1975) as well as heterologous antigens from simian malaria parasites such as *P. brasiliamim* (Mathews and Dilworth, 1976), *P. knowlesi* (Kagan, 1972, Lobel et al., 1973, Collins et al., 1975, Bagchi et al., 1978, Mahajan et al., 1981, Chandanani et al., 1981, Agarwal et al., 1982, Nath
P. cynomolgi antigen was found more superior than P. knowlesi antigen for the detection of P. vivax infection (Nath et al., 1984). More sensitivity and specificity of IHA was observed by using the homologous antigens as compared to the heterologous antigens (Lobel et al., 1973; Meuwissen et al., 1974, Mathews et al., 1975, Cornille-Brogger et al., 1978, Gupta et al., 1979, Ray et al., 1981, Chandanani et al., 1981). Antigen harvested from in vitro culture (Gupta et al., 1981, Ray et al., 1981) as well as lyophilized fixed erythrocytes (Meuwissen, 1974) were also employed successfully in IHA. Meuwissen et al. (1972) using various simian malaria antigens and human malaria patient sera, reported the various technical aspects of the haemagglutination assay for malaria with particular attention of the influence of heterophile antibodies (the antibodies reacting with antigenic determinants of noninfected heterologous erythrocytes) and in some infections the production of agglutinins against host erythrocytes components, so he advocated the absorption of sera with tanned sheep cells sensitized with noninfected host red blood cell antigen as a control on the IHA titre for specific agglutins.

In a comparative evaluation with other immunological assays, it was reported that in the younger age group (under 10 years), as well as during the initial infection, the immunofluorescence assay (IFA) detect the antibodies slightly more efficiently than the IHA, but in the adults and after several months of infection, the IHA tended to detect antimalarial antibodies more efficiently than IFA. The IHA also detected significantly higher malaria antibody titres in patients who had previously been infected with malaria (Wilson et al., 1971, Meuwissen, 1974, Meuwissen et al., 1974, Collins et al., 1975, Chandanani et al., 1981). Some other workers reported IHA in general somewhat less sensitive than ELISA and IFA (Mahajan et al., 1982, Agarwal et al., 1981, Khanna et al., 1991).

**IMMUNOFLUORESCENCE ASSAY**

Coons et al. (1942) for the first time reported that antibody could be labelled with fluorescent dye in such a manner that it retained its capacity to react specifically with the antigen. Since then, this finding has been applied for the immunospecific staining of a variety of microorganisms including various protozoans, such as *Entamoeba histolytica*, *Entamoeba coli* (Goldman, 1954), *Toxoplasmagondii* (Goldman, 1957), *Trypanosoma cruzi* (Fife and Mushel, 1959). The credit for the application of this technique in maliariology goes to Brooke...

The technique of IFA was found highly specific in infections with the genus *Plasmodium* and failed to reveal cross reactions with other infections such as trypanosomes, leishmaniids and ascarids (Kuvin et al., 1962, Voller, 1962, Ciucu et al., 1964), however this test is not found sufficiently specific to differentiate strains and species of plasmodia as the cross reactivity between different species of *Plasmodium* has been demonstrated by various groups of workers (Voller and Bray, 1962, Diggs and Sadun, 1965, Collins et al., 1966). The superiority of homologous antigen over the heterologous antigen has also been reported in IFA (Diggs and Sadun, 1965, Collins et al., 1966, Umgureanu et al., 1980).

Initially thin blood smears which required high parasitaemia were employed in IFA (Tobie and Coatney, 1961, Voller, 1962, McGregor et al., 1965) but subsequently use of thick blood smears at much lower parasitaemia have been used (Sulzer et al., 1969, Voller and O’Neil, 1971, Thomas and Ponnampalam, 1975, Mahajan et al., 1981, Agarwal et al., 1981). Collins and Skinner (1972) have employed hydrochloric acid (HCl) for the dehemoglobinization of blood smear but Voller (1975) reported the drop in IFA titres with the use of HCl. Modified immunofluorescence assay (MIFA), by using glutaraldehyde fixed and air dried monolayers of ring infected erythrocyte (RESA) of *P. chabaudi* (Gabriel et al., 1986), *P. fragile* (Nguyen-Denh et al., 1988) and *P. falciparum* (Perlmann et al., 1984, Deloron and Cot, 1990, Ruangjirachuporn et al., 1991) have also been reported. Antigen harvested from in vitro culture of *P. falciparum* have also been employed in IFA (Sodeinde
and Williams, 1990, Kumar et al., 1992). Rubik et al. (1989) used the lyophilized antigen prepared from continuous in vitro culture.

The comparative study of IFA with other immunological techniques such as IHA and ELISA for the serodiagnosis and seroepidemiological survey of human malaria has also been reported (Bray and Elnahal, 1966, WHO, 1974, Collins et al., 1975, Agarwal et al., 1981, Mahajan et al., 1981, Srivastava et al., 1991, Decarvalho et al., 1992).

**IN VITRO CULTURE OF ERYTHROCYTIC STAGES OF MALARIA PARASITES**

The investigations on immunological and other aspects of malaria require continuous supply of *Plasmodium* parasite in sufficient quantity. Due to less availability and difficulty in getting the suitable experimental hosts, the constant supply of malaria parasite remains a major problem among the malariologist. This led the scientists to attempt to cultivate malaria parasites in vitro system. Several attempts were made to establish the short term as well as continuous in vitro cultivation of malaria parasites and to develop in vitro assay systems. Bass and Johns (1912) for the first time reported the in vitro culture of human malaria parasites *P. falciparum* and *P. vivax* by incubating the whole defibrinated malaria patient blood supplemented with glucose at 37°C and observed the maturation of ring and sometimes the formation of new generation of invasive merozoites which rarely even leads to one or two additional cycles. During the subsequent years several attempts were made to see the effect of a variety of agents and different conditions such as temperature etc. on in vitro growth of the malaria parasites (Manwell and Hewitt, 1937, Hewitt, 1938). In these studies although the infectivity and survival of the parasite was observed for few days but no common agreement on conditions favourable to the in vitro growth of the parasite could be established. However, Trager (1941, 1943) has reported the first in vitro culture of *P. lophurae* up to 16 days and somewhat comparable results were obtained by Coulston (1941) for in vitro culture of *P. circumflexum* and by Hawking (1945) in the case of *P. gallinaceum*. Geiman et al. (1946) has employed two different techniques of rocker dilution and rocker perfusion for in vitro cultivation of *P. knowlesi* and obtained about 4 fold multiplication in 24 hr. Ball et al. (1945) and Anfinsen et al. (1946) while working at Harvard on in vitro growth of *P. knowlesi* in rhesus monkey erythrocytes described a synthetic nutrient medium (later known as Harvard medium) for in vitro growth of malaria parasites. Subsequently several groups of
workers have tried in vitro cultivation of erythrocytic stages of various species of Plasmodium using Harvard growth medium with different modifications and reported the effects of various components in the culture medium (McGhee and Trager, 1950, Geiman et al., 1966, Trager, 1971, Siddiqui and Schnell, 1973, Williams and Richards, 1973, Digs et al., 1975, Siddiqui et al., 1975, Trig, 1975). In most of these studies with the erythrocytic stages of both human and other nonhuman species of malaria parasites only one or at the most a few cycles of development in in vitro culture were observed, but even these short term experiments were found useful in accessing chemotherapeutic, biochemical and immunological studies. However, Trager (1976), after screening a number of different tissue culture media using P. coatneyi in rocker dilution flasks discovered that the medium RPMI-1640 (originally prepared by More et al. (1967) for the culture of human leucocytes) supplemented with 25 mM N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES buffer) was significantly superior than the Harvard medium. Finally two groups of workers namely Trager and Jensen (1976) and Haynes et al. (1976) independently reported the successful continuous in vitro cultivation of erythrocytic stages of P. falciparum. Trager and Jensen (1976) have employed RPMI (Rosen Park memorial Institute) -1640 medium supplemented with HEPES buffer (25 mM) and 2% NaHCO$_3$ with 10% human type AB serum either using a continuous flow vial method in an atmosphere of 7% CO$_2$, 1 or 5% O$_2$ and 92-88% N$_2$ or using a simple candle jar method producing an atmosphere of low O$_2$ and high CO$_2$ content incubated at 37° and normal change of medium daily. Haynes et al. (1976) have used the modified tissue culture medium-199 (with Earle's modified salts), enriched with glucose (2 mg ml$^{-1}$), 2 mM glutamine, 3x10$^{-5}$M 2-mercaptoethanol, (±)α-tocopherol (emulsified) (30 μg ml$^{-1}$), plus 10% heat inactivated foetal bovine serum and 10 mM N-Tris-(hydroxymethyl)-methyl-2-aminoethane sulphonate (TES) buffer, using culture flask/microtitre plates in an atmosphere of 3% CO$_2$, 6.6% O$_2$ and balanced nitrogen at 37°. Subsequently Jensen and Trager (1977) have reported in vitro cultivation of P. falciparum using petridishes held in candle jar with manual change of medium daily. This method known as petridish-candle jar method is the most widely used method for in vitro cultivation of the blood stages of malaria parasites. Later on several groups of workers successfully developed in vitro cultivation of P. falciparum in RPMI-1640 medium, using the gas mixture or candle jar by employing the basic techniques of Trager and Jensen (1976) or with some modifications, such as using petridishes or culture

Although Larrouy et al. (1981) have reported the culture of P. vivax for 43 days by using essentially the similar method of Trager and Jensen (1976) except using higher glucose level and change of medium thrice daily rather then once, however in several other attempts to adapt the P. falciparum culture system to P. vivax no significant success could be achieved (Siddiqui, 1979, Chowdhury et al., 1982, Renapurkar et al., 1983, Brockelman et al., 1985, Mons et al., 1988). Recently Lanners (1992) reported the prolonged in vitro cultivation of P. vivax using Trager’s continuous flow method.

The in vitro culture of several species of simian plasmodia such as P. knowlesi (Butcher, 1979, Wickham et al., 1980), P. cynomolgi (Nguyen-Dinh et al., 1981, Zhaoxi et al., 1984), P. fragile (Chin et al., 1979) and rodent malaria parasites such as P. berghei (Guru and Sen, 1980, Mons et al., 1983) P. chabaudi (Mclean et al., 1986) have also been established by employing the basic technique of Trager and Jensen (1976) or with some modifications.

The various species of Plasmodium were cultured using erythrocytes obtained from their natural hosts, such as simian species of Plasmodium have so far only been successfully cultured in erythrocytes from rhesus (Macaca mulatta) or kra (M. fascicularis) monkeys, while human erythrocytes were considered to be suitable for continuous culture of P. falciparum, however the in vitro culture of P. falciparum in Aotus trivirgatus erythrocytes has also been reported (Peterson et al., 1984). Jensen and Trager (1977) reported that erythrocytes from blood stored in acid citrate dextrose (ACD) or citrate-phosphate dextrose (CDP) until they were outdated for blood banking purposes may still be used for in vitro culture of P. falciparum. Capps and Jensen (1983) reported that the erythrocytes stored for 84 days in adenine supplemented CDP were still suitable for P. falciparum culture.

The problem of acquisition of sufficient quantity of human serum on regular basis and
moreover the possibility of its contamination of hepatitis viruses and other pathogens and presence of possibly inhibiting malarial antibodies and chemotherapeutic agents specially in malarious region, led to the attempts to replace the human serum with serum obtained from a variety of animals such as horse, bovine, calf, swine, lamb, sheep, rabbits etc. and successful in vitro culture of *P. falciparum* is reported (Jensen, 1979, Butcher, 1979, Ifediba and Vanderberg, 1980, Siddiqui 1981, Raichowdhury and Sivaraman, 1982, Divo *et al.*, 1985, Grun and Weidanz, 1987). The in vitro culture of *P. falciparum* upto four weeks or longer has also been reported in serum free RPMI-1640 medium supplemented with adenosine, fatty acid-free BSA and oleic or cis-vaccenina acid (Willet and Canfield, 1984).

Different methods were applied for the synchronization of parasites in vitro culture such as percoll gradient centrifugation (Ruangjirachuporn *et al.*, 1991), by treating with D, L-α-difluoromethylornithine (Assaraf *et al.*, 1986) or by Sorbitol treatment (Lambros and Vanderberg, 1979, Read, 1992). Hoppe *et al.* (1991) reported a comparative study of different synchronization methods for in vitro culture of malaria parasites.


Trager and Williams (1992) reported the extracellular (axenic) development of atleast
one complete erythrocytic cycle of *P. falciparum* in *in vitro* using 24 well culture plate and candle jar by suspending the erythrocytic merozoite in a medium of sonicated erythrocyte having ATP, pyruvate and mixed with matrigel to form a soft gel, which is overlaid with complete liquid medium-KF, a high potassium modification of RPMI-1640 medium, that is replaced with fresh medium at appropriate intervals of 12, 24 and 36 hr. At all these times and also at 45 hr rhodamine 123 was added to same culture and gels were sampled. They observed the viable extracellular forms showing rhodamine fluorescence: rings at 12 hr, trophozoite and early schizonts with pigment at 36 hr and late schizonts with developing merozoites at 45 hr. These merozoites were found infective to erythrocytes added to the culture at 45 hr. Moreover in the electron micrograph of 36 hr trophozoite, only single plasma membrane is observed and no parasitophorous membrane is seen. Thereby they concluded that for the development of merozoite through it complete asexual cycle, the complex process of its entry into erythrocyte, the intactness of the host erythrocyte and the parasitophorous membrane are not essential.

**ISOLATION AND CHARACTERIZATION OF MALARIAL ANTIGENS**

Malaria parasites are having a complex mixture of antigenic components having both common or cross reactive as well as stage, species and strain specific antigens (Collins *et al.*, 1966, Deans *et al.*, 1978, Kilejian, 1980, Miller *et al.*, 1980, Brown *et al.*, 1982b, Kaushal *et al.*, 1990, Moelans and Schoenmakers, 1992). Total cell free extracts therefore need to be analysed into component antigens in order to focus attention on those antigens which are potent immunogenic and to identify and characterize any particular cross reactive or specific antigen. So it will provide more rational basis for developing effective malarial vaccine as well as specific immunodiagnostic test. Number of techniques based on immunoprecipitation and immunoelectrophoresis in agar gel system such as double diffusion (Ouchleroney, 1958), immunoelectrophoresis in agar gel system (Graber and Williams, 1953), immunoprecipitation of metabolically labelled antigens (Epstein *et al.*, 1981), SDS-polyacrylamide gel electrophoresis (Laemmli, 1970), autorgraphy, fluography and several other chromatographic techniques have also been employed in the field of malaria for the analysis, identification, isolation and characterization of plasmodial antigens.

The analysis and isolation of the antigens from the parasite and from the host
erythrocyte membrane requires the solubilization of the collected parasites and separation of
the soluble from the insoluble materials by extraction and centrifugation. Extraction buffer
containing a cocktail of protease inhibitors (in order to protect the antigens from the attack
of the proteases and other digesting enzymes, so as to get the isolated antigens as intact as
possible) and a number of nonionic or ionic detergent such as Lubrol, various Tritons,
Nonidet NP 40, Desoxycholate or Zwittergent 314 etc. were employed for solubilizing the
parasite components (Leech et al., 1984, Howard and Barnwell, 1984). The nonionic
detergent Triton X-100 or Berol EMU-043 has been found suitable as it causes minimal
protein denaturation (Bjerrum and Lundall, 1975, Bjerrum et al., 1975, Deans et al., 1978,
Kaushal et al., 1983).

In some of the earliest attempts, the presence of both species specific and cross reactive
antigens between the rodent malaria parasites *P. vinckei* and *P. berghei* has been reported
by applying the technique of immunoelectrophoresis in agar gel system (Spira and Zuckerman,
1966). Subsequently by applying the techniques of double diffusion and immunoelectrophoresis,
the antigenic analysis of various species of plasmodia such as *P. lophura* (Sherman, 1964),
*P. gallinaceum* (Smith et al., 1969), *P. berghei* (Diggs, 1966, Zuckerman et al., 1969, Steitz,
1972), *P. cynomolgi* (Banki and Bucci, 1964), *P. knowlesi* (Brown et al., 1970b), *P.
falciparum* (Mcgregor et al., 1966, Turner and McGregor, 1969a,b, Wilson, 1970, Wilson
et al., 1969, 1975a,b, 1976) were reported. Sherman (1964) has reported the antigenic
analysis of the avian malaria parasite *P. lophurae* by double diffusion and immunoelectrophoretic
techniques and demonstrated the presence of haemoglobin as one of the major antigenic
components as a contamination in parasite antigenic preparation. Smith et al. (1969) identified
a soluble antigen and its specific antibodies in the sera of chicken infected with *P. gallinaceum*
by employing the techniques of double diffusion and immunoelectrophoresis. Diggs (1966)
studied the antigenic analysis of rodent malaria parasite *P. berghei* by employing the
technique of double diffusion and immunoelectrophoresis using hyperimmune rabbit serum
and drew attention to the presence of antibodies against antigens of host origin such as
haemoglobin and other serum components and emphasize that their presence must be taken
into consideration in accessing the results. In another study Zuckerman et al. (1969) by
employing the double diffusion in agar gel system reported the kinetics of the production of
precipitin lines in one hundred and eleven samples of the rat anti-*P. berghei* sera obtained
during the primary infection and found the 55% precipitin positive reaction. A series of subsequent eight times hyperimmunization by reinoculation of viable *P. berghei* raises the percentage of positive test from 55% to 75% while the vaccination with cell free plasmodial product before challenge with viable parasites raises the percentage of positive test to 100%. The species specificity of at least part of the precipitins was demonstrated but no antierythrocytic precipitin was recognized. Steitz (1972) has employed the techniques of double diffusion and immunoelectrophoresis to demonstrate the presence of soluble antigens in the sera of mice infected with *P. berghei*. Brown et al. (1970b) have employed the double diffusion technique for analysing the antibody response in the anti-*P. knowlesi* rhesus monkey sera and reported the appearance up to three precipitin lines with sera having chronic infection and four strong precipitin lines with sera from vaccinated monkey against *P. knowlesi* schizont extracts, some lines were found strain specific while others were common to various strains of *P. knowlesi*. McGregor et al. (1966) has also employed the technique of gel diffusion for analyzing the sera collected from individuals exposed to *P. falciparum* in Gambia. In other studies by applying the technique of gel diffusion two kinds of antigens: α-antigens (mol. wt. 300-900 KD) and β-antigens (mol. wt. 50-60 KD) in the extract of *P. falciparum* infected placental blood were reported (Turner and McGregor, 1969a,b). Wilson et al. (1969, 1973) by employing the technique of gel diffusion using the anti-*P. falciparum* sera from Gambian individuals detected the various *P. falciparum* soluble antigens in the plasma of many individuals (mostly children) with acute infections and other antigens from the infected blood cells and typified them according to their thermostability into heat labile ‘L’ (with subclass Lα1, Lα2, Lb), heat resistant ‘R’ (with subclass, R1, R2) and heat soluble ‘S’ groups of antigens. Wilson (1970) in a separate study reported the considerable degree of common antigenicity among these ‘S’, ‘L’ and ‘R’ groups of *P. falciparum* antigens from the two widely separated area of Gambia and Nigeria in West Africa. These ‘S’, ‘L’ and ‘R’ groups of antigens were also reported in the peripheral blood of *Aotus* monkey infected with *P. falciparum* and their antigenicity were compared with antigens obtained from the human source by employing the gel diffusion technique (Wilson and Voller, 1972). Subsequently the technique of gel diffusion has also been employed to study the frequency of the occurrence of soluble ‘S’ antigen in the sera of individuals having *P. falciparum* infection following antimalarial treatment and during subsequent parasitic episodes in the same individuals (Wilson et al,
In another study, Wilson et al. (1976) successfully detected precipitating antibodies to malaria 'S' antigens by gel diffusion in the sera from young Gambian children with acute *P. falciparum* malaria.

Spira et al. (1966) have reported the application of a sensitive disc gel electrophoresis technique for analysing the protein constituents of different species of *Plasmodium* such as rodents (*P. berghei, P. vinckei*), chicken (*P. gallinaceum*) and monkeys (*P. knowlesi, P. cynomolgi-B*) and found the difference in the relative positions of protein bands in the electrophoretic patterns of various plasmodia. Smith et al. (1969) employed the disc gel electrophoresis technique for the characterization of soluble antigen and its antibody in the sera of chicken infected with *P. gallinaceum*. Steitz (1975) has employed a more sensitive, rapid and simple technique of counter current immunoelectrophoresis using cellulose acetate membrane as matrix for the detection of malarial antigen and antibodies in the sera of mice and rats having *P. berghei* or *P. vinckei* infections.

One of the most sensitive technique for analysing the antigens and antibodies is the cross immunoelectrophoresis (CIE). Bjerrum and BOG Hansan (1976) employed this technique for the characterization of the erythrocyte membrane proteins immunochemically. SDS-PAGE is the widely used technique for analysis, identification, characterization and even semipreparative isolation of proteins (Laemmli, 1970). Subsequently, the techniques of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and CIE have been successfully employed by several groups of workers for analysing the protein and antigenic constituents of the erythrocytic stages of the various species of rodent (Carlsson et al., 1984, Panton et al., 1984a,b) and simian (Butcher et al., 1978, Deans et al., 1978, Deans and Cohen, 1979, Schmidt-Ullrich et al., 1979, Miller et al., 1980, Epstein et al., 1981, Anders, 1985, Haider, 1990) malaria parasites. The antigenic composition of different erythrocytic stages of *P. knowlesi* has been analysed by employing the technique of CIE using a pool of immune rhesus monkey IgG and the presence of nine stage independent (shared by rings, trophozoites, schizonts and merozoites) and two stage specific (one specific to trophozoite and other was restricted to mature schizont and merozoite) antigens out of the eleven major parasite antigens were identified (Deans et al., 1978, Deans and Cohen, 1979). Schmidt-Ullrich and Wallach (1978) detected three *P. knowlesi* schizont specific antigens out of the seven parasite specific antigens identified in the membrane of the parasitized erythrocytes by employing the
techniques of CIE and CLIE using monkey anti-\textit{P. knowlesi} schizont serum. In another study Schmidt-Ullrich \textit{et al.} (1979) immunochemically analysed the two strains of \textit{P. knowlesi} using IgG fractions of monkey anti-\textit{P. knowlesi} schizont hyperimmune serum and sera from naturally immune monkeys and found that only two out of the >20 antigenic components in the Triton X-100 solubilized schizont infected erythrocytes of \textit{P. knowlesi} were recognized during the course of natural infection. Panton \textit{et al.} (1984a,b) have identified a strain specific antigen of \textit{P. yoelii} (Py-1) by employing the technique of CIE. Carlsson \textit{et al.} (1984) analysed the Triton X-100 extracts of the erythrocytes infected with \textit{P. chabaudi} and \textit{P. yoelii} by CIE using rabbit antisera, about 12 immunoprecipitate were obtained with both the extracts and their homologous antiserum and the cross test showed that two species of plasmodium are very similar antigenically, however the CLIE with intermediate gel containing the heterologous antiserum demonstrated the presence of atleast one antigen specific for each of the parasite extracts.

The metabolic radio labelling techniques have also been employed for the identification and isolation studies of malaria antigens. Radiolabelled amino acids incorporated in proteins are traced in this technique. \textsuperscript{14}C, \textsuperscript{35}S or \textsuperscript{125}I amino acids are generally used for labelling proteins. \textsuperscript{14}C glucosamine or \textsuperscript{13}C sugars are used for labelling parasite glycoproteins whereas radioactive fatty acids such as palmitic acid are used to label lipids or glycolipids (Heidrich, 1988). Radiolabelling are carried out in \textit{in vitro} culture using special cultural medium having particular labelled constituents. For successful labelling the selection of appropriate time during the life cycle of the parasite is also important (Venot-Hernandez and Heidrich, 1984). For labelling the surface proteins of infected erythrocytes, merozoites or other cells \textsuperscript{125}I labelled amino acids are mostly used. Among the various methods of labelling such as iodogen procedure (Markwell and Fox, 1978, Heidrich \textit{et al.}, 1983), iodobead procedure (Markwell, 1982), the lactoperoxidase procedure (Phillips and Morrisen, 1971, Heidrich \textit{et al.}, 1983) is found most effective. Some proteins can only be labelled with a specific amino acid and will not be traced when other radiolabelled amino acids are used, for example a protein or a histidine rich protein will be more easily found when proline or histidine label is used (Hadley \textit{et al.}, 1983). The detergent extracts from parasites containing radiolabelled antigen may then be immunoprecipitated by reacting with immune sera either from patients, from endemic area or from hyperimmune animals or with monoclonal antibodies and the immune
complex formed may further be analysed by SDS-PAGE, autoradiography and fluorography (Howard et al., 1983).

Attempts were also made to fractionate plasmodial extracts in order to isolate and purify any individual antigen. Plasmodial extracts from human (Mahoney et al., 1966, Sadun and Gore, 1968, Turner and McGregor, 1969a), Simian (Cook et al., 1969) and rodent sources (Chavin, 1966, Wellde et al., 1969, D’Antonio et al., 1970) have been fractioned. A variety of fractionation procedures have been employed such as centrifugation and extraction (Mahoney et al., 1966, Cook et al., 1969), salting out (Wellde et al., 1969), Disc electrophoresis (Hamburger and Zuckerman, 1976a,b) and various chromatographic procedures such as molecular sieving (D’Antonio et al., 1970, Turner and McGregor, 1969a, Wilson et al., 1969, Wilson and Ling, 1979, Strycher et al., 1986), Ion exchange chromatography (Wellde et al., 1969, Sadun et al., 1969, McAlister, 1972, Saxena et al., 1988) and affinity chromatography (Jungery et al., 1983, Wahlin et al., 1990).

**THE PROSPECTS OF MALARIA VACCINE**

During the recent years attempts has been focussed to immunize against purified antigenic components as candidate vaccine from different stages of *Plasmodium* in order to induce specific protective immunity (McGregor, 1988, Phillips, 1992) and efforts were done to develop malaria vaccine based on the humoral mediated immunity as well as the vaccine strategy based on T cell and the induction of cell-mediated immunity (Cattani, 1989, Good, 1992, Good et al., 1992, Romero, 1992, Diggs, 1992, McGregor, 1993) against different stages of malarial parasite such as sporozoite (Chulay, 1989, Good, 1990, Hoffman et al., 1991, Herrington, 1991), liver stage (Mazier et al., 1990, Cox, 1992), merozoite (Sharma et al., 1992, Kumar et al., 1992a, Copper, 1993), intraerythrocytic stage (Butcher, 1989, Moreno and Petarroyo, 1989, Weidanz et al., 1990, Long, 1993) as well as sexual gamete stage (Targett, 1990, Kaslow 1990, 1993). Attempts has also been done to develop antitoxin vaccine i.e. the toxic substance released by the parasite when the infected RBCs rupture and it may be neutralize by inducing specific immunity (Playfair et al., 1990). The vaccine strategy based on T cell and cell mediated immunity (Special issue, 1990, Quakyi et al., 1992, Hviid et al., 1992, Waki et al., 1992) involves the role of macrophages, granulocytes (Ferrante et al., 1990, Turrrini et al., 1992, Schwarzter et al., 1992), natural killer (NK) cells, MHC and HLA (Riley et al., 1992, Hill et al., 1992), C-creative protein
(Chagnon, 1992), and number of cytokines (Vreden et al., 1992, Toverne, 1993), such as gamma interferon (Maheshwari et al., 1990), tumour necrosis factor (Clark et al., 1990, Clark, 1992) etc.

Out of the different antigenic component tried only three malaria vaccine candidate antigens were used for the clinical trials in human volunteers. Two of them were based on the immunodominant epitopes of the circumsporozoite proteins, the main component of the surface coat of malaria sporozoite: one consisted of a 12 amino acid synthetic peptide (NANP), (Herrington et al., 1987) and other was a recombinent polypeptide containing the NANP repetition 32 times and linked to a 32 amino acids of the tetracycline resistance gene: R 32 tet 32 (Ballou et al., 1987). The third vaccine which is used in the III phase clinical trial is the blood stage synthetic vaccine ‘SPf 66’, consisted of three merozoite specific peptides (35 KDa, 55 KDa and 83 KDa) hybridized into a 45 amino acids peptide, devised by Columbian biochemist Dr. Mannuel Patarroyo who after a successful trial in owl monkey Aotus trivirgatus (Patarroyo et al., 1987) finally tried in human volunteers using aluminium hydroxide as adjuvant and again found a very promising result with 80% protection (Patarroyo et al., 1988). But in other independent trials conducted at Centre for Disease Control (CDC) in Atlanta, USA (Ruebush et al., 1990) and in Columbia (Herrera et al., 1992), the results of Patarroyo could not be reproduced. Though the Patarroyo attributed the failure of the other groups to the problem of the peptide-BSA conjugation process (Patarroyo, 1990), but the Patarroyo vaccine came in skepticism and his work was criticized on several grounds such as, the extent of malarial endemicity in some study regions was not certain and his human trials were not critically controlled and contained no placebo groups, experiments were not randomised and double blind and the malaria infection rates among the unvaccinated controls were low, so the efficacy of Patarroyo’s SPf 66 vaccine remained doubtful and even the Britain’s Medical Council (MRC) refused to sanction a further randomised double blind protocol for trial in Gambia in the Western Africa, a highly endemic area of P. falciparum, on the ground that the available technical data on Patarroyo’s vaccine were not adequate to justify the MRC support for the experiment in humans (Cherfas, 1990b, Brown, 1991, 1992a). Despite all these setbacks Patarroyo’s group continued his field trials and finally came up with an improved version of SPf 66 vaccine (now consisted of three merozoite specific peptides (35 KDa, 55 Kda and 83 Kda), hybridized to a 45 amino acids peptide and with an
extra tetrapeptide sequence from a CS protein and then polymerised to form a synthetic vaccine) and again with a claim of 80% protection rate against *P. falciparum* (Salcedo *et al.*, 1991, Amador, 1992b, Patarroyo, 1992). After a lot of controversies and skepticism, the Patarroyo’s SPf 66 vaccine has now been taken seriously by World Health Organisation and many other human trials are in progress and their results will soon decide the efficacy and future of Patarroyo’s vaccine (Brown, 1992b, Marshal, 1992, Cox, 1993).

The slow progress of malaria vaccine development may be attributed to the complex life cycle of the parasite, its changing morphology and the nature of its interaction with its host. During malarial infection the immune defence mechanisms of the host and immune evasion mechanisms of the malarial parasite remain interplay with each other. The parasite has evolved number of strategies to evade the host immune response such as antigenic variation, antigenic polymorphism, antigenic mimicry and direct interference with the generation of the host immune response (Pereira de Silva, 1990, Biggs *et al.*, 1991, Mendis *et al.*, 1991, Kumaratilake and Ferrante, 1992, Theunder, 1992, Howard, 1992).

**THE PROSPECTS OF DIAGNOSIS OF MALARIA**

Malaria is often an underdiagnosed and neglected medical emergency (Oliver *et al.*, 1991, Rougment *et al.*, 1991, Cook, 1992, Van Den Ende *et al.*, 1992). The criteria for detection and diagnosis of malaria include: sensitivity, specificity, simplicity in application, unambiguous interpretation and rapid turn around time (Pammenter, 1988, Payne, 1988). Presently the differential Giemsa stained thick and thin blood smear, examined under the microscope remains the most reliable and definitive test for the diagnosis of malaria (Shute, 1988, Makler and Gibbins, 1991). The fluorescent dye benzothiocarboxy purine (BCP) has also been employed for the microscopic detection of *P. falciparum* and it was found more better and advantageous than the standard Giemsa stain (Makler *et al.*, 1991, Cooke *et al.*, 1992).

Recently, a rapid and more sensitive acridine organe fluorescent microscopy using the quantitative buffy coat capillary (QBC) tube was employed for the detection of plasmodia (Levine *et al.*, 1989, Rickman, 1989, Wongsrichanalai *et al.*, 1991, Poggensee *et al.*, 1992), but in the field trial it met several difficulties and also found less sensitive than the conventional thick and thin blood smear Giemsa staining method (White and Silamut, 1989, Baird *et al.*, 1992).

Recent studies on the parasite genome (Weber, 1988, Triglia *et al.*, 1992) and progress
in the field of biotechnology led the production of pure malarial antigens by genetic manipulation (Scaife et al., 1986, Godson, 1986) and specific monoclonal antibodies by hybridoma technology (Kamber et al., 1992) which may be used for the development of alternate diagnosis tests to microscopy based on molecular detection and immunodetection of malaria (WHO, 1988b, Nantulya, 1991).


Enzymes have also been exploited for the diagnosis of malaria, such as by plasma lactate dehydrogenase (LDH) estimation (Klenerman et al., 1992), by measuring the activity of parasite LDH (Makler and Hinrichs, 1993) and by detecting parasite LDH using plasmodium specific anti-LDH antibodies by immunodot enzyme staining test (Kaushal, 1993).
SCOPE AND PLAN OF WORK

The conventional methods available for malaria control based on insecticides such as dichlorodiphenyl trichloroethane (DDT) to kill the vector, and anti-malarial drugs, such as chloroquine to treat the infected individuals are steadily declining in efficacy, mainly due to the development of insecticide resistance in mosquitoes and drug resistance in malaria parasites, specially in Plasmodium falciparum. This situation has resulted in the widespread resurgence of malaria even in the areas from where once it had vanished. The chloroquine and multiple drug resistant strain of P. falciparum is increasingly spreading in many areas of the world including India. This grim scenario led the scientists and research organisation such as WHO not only for finding the better chemotherapeutic agents but also towards finding an immunological solution of the disease such as the development of effective malarial vaccine to supplement the existing and future malaria control strategies.

The development of malarial vaccine as well as better immunodiagnostic methods are the two major objectives of the scientific working group (SWG) of WHO on the immunology of malaria (IMMAL) since it was set up in 1976. During the recent years there has been considerable efforts for developing effective malarial vaccine against different developmental stages of malarial parasites viz. sporozoite, asexual blood stages as well as gametes. However, the asexual blood stages remain the main focus of malarial research on vaccine development and immunodiagnostics as the morbidity, its clinical symptoms and mortality due to malaria is mainly caused by these stages. Therefore the research in this direction will be of special importance particularly for the tropical and subtropical regions of the world and certainly for India.

The morphological and biological similarities between human and nonhuman primate malarias makes the later good animal model for human malarias providing suitable laboratory host available. So the work reported in this thesis is directed towards the immunochemical studies on asexual blood stages of Plasmodium knowlesi, a simian malaria parasite and P. falciparum (the human parasite) and deals with the following aspects:

(1) IMMUNOGENICITY AND PROTECTIVE POTENTIAL OF PLASMODIUM KNOWLESI SCHIZONT INFECTED ERYTHROCYTES

1.1 Isolation and purification of Plasmodium knowlesi schizont infected erythrocytes (PK SI-RBC)
1.2 Immunization of rabbits against *P. knowlesi* SI-RBC and quantitation of parasite specific antibodies in hyperimmune rabbit sera.

1.3 *In vitro* evaluation of rabbit anti-*P. knowlesi* SI-RBC sera for protective potential of antibodies.

(2) **PROTEIN AND ANTIGENIC ANALYSIS OF *P. KNOWLESI/P. FALCIPARUM* SCHIZONT INFECTED ERYTHROCYTES**

2.1 Protein pattern of *P. knowlesi* SI-RBC.

2.2 Antigenic analysis of *P. knowlesi* SI-RBC.

2.3 Identification of common or cross reactive antigens of *P. knowlesi* and *P. falciparum* SI-RBCs.