GENERAL INTRODUCTION

Introduction to malaria

From time immemorial malaria has been one of the most prevalent of human diseases, affecting particularly the population of tropical regions but also in the past those of temperate climates. Inspite of decades of efforts at its control, malaria continues to rank as one of the foremost infectious global diseases. Over two billion people, half of the world's population, are exposed to the risk of the disease and about 270 million acquire new infections each year (Peters 1990a). According to World Health Organization (WHO) malaria kills every year between 1.4 to 2.8 million people, mostly children in Africa (TDR news 1994).

Causative agent

Malaria is caused by a protozoan parasite of genus Plasmodium. The genus belongs to phylum Apicomplexa (formerly Sporozoa), of the animal subkingdom Protozoa (Mehlhorn and Walldorf 1988). All members of the phylum are parasitic. Plasmodium species exhibit two types of schizogony (asexual multiplication), an exoerythrocytic schizogony without pigment formation and an erythrocytic schizogony with pigment formation, in the chordate host and a sexual stage terminating in sporogony in the female mosquito host. They are found infecting mammals, particularly primates and rodents, birds and reptiles. Systematic position of malaria parasites is as follows:

- **Phylum**: Apicomplexa
- **Class**: Sporozoa
- **Subclass**: Coccidia
- **Order**: Haemosporida
- **Suborder**: Aconoidina
- **Family**: Haemosporidae
- **Genus**: Plasmodium
with increase in parasitemia with each cycle, until it is impeded by drugs or the host defence mechanisms. The gametocytes persist in the circulation for a limited period of days and degenerate, if not picked up by an appropriate mosquito species for their further development in the gut of the new host. After arrival in the midgut of the mosquito the mature viable male gametocyte (microgametocyte) undergoes an explosive transformation called exflagellation which gives rise to 8 motile microgametes. The microgametes set themselves free in search of a female gamete (macrogamete), one arising out of each mature viable macrogametocyte. Fusion of a micro and a macrogamete in the midgut of mosquito results in the formation of diploid zygote. This stage transforms into an actively moving form called ookinete stage and penetrates the midgut wall. The successful ookinete lodge themselves inside the wall and round themselves up to transform into oocysts. Meanwhile reduction division has occurred following recombination and haploid genome is restored. The oocyst undergoes repeated mitotic divisions followed by development and differentiation and release of thousands of infective sporozoites into the hemolymph of the mosquito. These sporozoites find their way into salivary glands of the host and are ready for initiating a new infection during the next bite of the vertebrate host.

**Rodent malaria parasite**

The first isolation of rodent malarial parasite was reported by Vincke and Lips (1948) from the African tree rat, *Thamnomys surdaretis* and was named *Plasmodium berghei*. Since then numerous species and subspecies of rodent malaria have been discovered and described. They have been divided into two main series, the berghei group, consisting of *P. berghei*, *P. yoelii* and *P. y. nigeriensis* and other subspecies and the vinckei group consisting of *P. vinckei* and *P. chabaudi*, which also have several subspecies. About two hundred different strains of *P. berghei* have been isolated from various rodent or from the natural vectors. *P. berghei* has superseded the bird malarial, *P. lophurae* and *P. gallinaceum*, in the utility for
experimental work on parasitism, immunology and chemotherapy of malaria. Dozens of papers on these various subjects are being published every year pointing out the importance of rodent malaria in scientific research and most of the primary screening of some 300,000 compounds developed during the past ten years was performed using rodent plasmodium (Landau and Boulard 1978).

**Human malaria parasite**

*P. falciparum, P. vivax, P. malariae and P. ovale* are the four species of malaria parasite known to infect humans. Different species predominate in different geographic regions, although transmission patterns can change with time. For example, *P. vivax* was (and remains) the major species transmitted in parts of India, but in recent years there has been resurgence of *P. falciparum* transmission in these areas. In Africa, *P. falciparum* predominates and *P. vivax* is virtually absent (most Africans lack the complement of Duffy blood group substances that define susceptibility to this parasite species) (Miller et al. 1976). *P. malariae* the second most common species in Africa is also distributed in other geographic regions. *P. ovale* is the least common species in Africa.

**Disease symptoms and pathogenicity**

Malarial paroxysms generally develop within a few days of inoculation of infective sporozoites. They consist typically of sequential chills, fever and sweating, severe headache, abdominal pain, nausea and vomiting. Attacks coincide with parasite multiplication in the red blood cells. As the parasite population in the blood increases, the symptoms intensify, subsiding only when infection is terminated by appropriate drug treatment or controlled by the host's immune system. *Falciparum* malaria is the most dangerous form of the disease. Two important biological features have been identified and implicated for its lethal potential. First, asexual blood stage parasites can infect and develop in erythrocytes of all ages, so there is no intrinsic limit on the potential magnitude of the parasitemia
High parasitemia results in severe hemolysis and its attendant complications. In contrast, \textit{P. vivax} and \textit{P. ovale} can develop only in young erythrocytes (Pasvol and Wilson 1982), thus placing a relatively a strict limit on the maximal magnitude of the parasitemia. \textit{P. malariae} is also restricted to a subpopulation of erythrocytes, probably older ones (Pasvol and Wilson 1982). Another distinctive feature of \textit{P. falciparum} is that electron dense extruberances, called knobs, develop on the surface of erythrocytes containing mature trophozoites or schizonts (Langreth et al. 1978). These knobs mediate binding of the infected cells to post capillary venules, where the parasites undergo their terminal maturation (called deep-vascular schizogony) and where the rupture of schizont infected cells finally occurs. As a result of this sequestration, mature intraerythrocytic forms are rarely detected in blood smears of patients with falciparum malaria. The combination of severe anemia (due to hemolysis of large number of infected erythrocytes) and microvascular congestion that results from deep vascular schizogony gives rise to tissue hypoxia and subsequently organ dysfunction; the brain, kidneys and lungs are particularly susceptible. Although vivax, ovale and malariae infections are not generally life threatening, they can sometimes cause severe, acute illness.

**Diagnosis and detection of malaria parasite**

Malaria diagnosis is confirmed by the detection of the causative agent in the blood of an infected individual. Microscopic detection of the parasite has been the mainstay of diagnosis in epidemiological surveys. The method is sensitive enough to detect a parasitemia as low as 0.002% in a thick film using as little as 0.2 to 0.5 \( \mu \)l of blood (Bruce-Chwatt and Zuluera 1980). Even 20 to 40 parasites \( \mu \)l of the blood can be detected (on an average 1 \( \mu \)l of blood contains over five million cells). For the detection of an individual case of malaria the amount of information obtainable by conventional light microscopy is remarkable. It allows simultaneous identification of the plasmodial species and the stage of the parasite.
(Field et al. 1963). It also permits rapid estimation of the level of parasitemia, an assessment of the degree of concomitant anemia and the presence of other blood parasites— all within less than 30 minutes, including staining time. However, this technique requires competent, committed and conscientious microscopist for correct diagnosis. It has often been the experience of many malariologists that due to human error quite a few cases are erroneously diagnosed, this being more so during peak malaria season and specially during the epidemiological surveys. This shortcoming in the use of simple, inexpensive microscopic method calls for alternate approaches for detection of malaria which not only take care of this problem but could also tell the susceptibility status of the parasite to the antimalarial drugs. Utility of various serological and immunodiagnostic methods has been explored but they have proved to be of limited value as the detection is possible only if the immune response has risen to a certain level. Also sometimes it is difficult to differentiate between past and present infections (Bruce-Chwatt 1987). Another promising approach being pursued to diagnose malaria is based on the use of nucleic acid probes. Identification of unique characteristic nucleic acid sequences in the genome of parasite species and presence of these sequences in the blood samples is determined using complementary nucleic acid sequences, tagged either with radiolabeled or non-radiolabeled compounds, as probes. Hybridization of these probes to the corresponding sequence of the pathogen is monitored by the signals emitted by the linked tags. This is a highly specific and sensitive method. This technique, involving synthetic oligonucleotide probes, has been successfully employed in field conditions in Kenya where parasitemia of 0.002% of infected erythrocytes could be detected in 1 μl of blood sample (McLaughlin et al. 1987). Most of the work on probe developments has been on *P. falciparum*. Recently Snounou et al. (1993) have reported the use of polymerase chain reaction (PCR) for the identification of the four human malaria parasite species. They used synthetic oligonucleotide primer pairs which were complementary to genus and species specific sequences
present within the small subunit ribosomal RNA genes of the four human malaria parasites, for the specific amplification by PCR to detect each malaria species. Thus the present methods of malaria parasite detection range from microscopic examination of blood smears, immunodiagnostic/serological approaches, DNA based probe hybridization to in vitro amplification of specific nucleic acid sequences of the pathogen. Of these, light microscopic method is the most popular and will continue to play an important role in confirming the malaria diagnosis even in near future for its sensitivity and simplicity. However, there exists a lot of scope for future improvements in the detection techniques.

**Malaria control**

It is certainly frustrating to observe that inspite of considerable investments in term of human efforts and money spent during the last two decades or so, no effective useful vaccine against human malaria has emerged and its control still depends on breaking any of the several links in the chain of parasite development through man and mosquito. Vector control and chemotherapy are the two most important tools to control malaria.

**Vector control**

Human malaria is transmitted by various species of *Anopheles* mosquito. Malaria transmission occurs in sub-Saharan Africa, parts of Asia, many countries in Latin America and small transmission foci also exist in Greece, Turkey and the Middle East (Wyler 1993). It is well known that epidemiology of malaria in any region is influenced by the geographical, climatological conditions, type of vector species- its bionomics, flight range, density etc., strains of parasites and immunological status of the human population. Malaria, therefore, is acknowledged as an exclusively local phenomenon and any control programme has to be meticulously designed depending upon the local situation and available resources. Early attempts to control malaria vectors were directed at the immature aquatic stages in their breeding places and involved either the use of larvicides or aimed at source
reduction. It might be a method of choice in some urban areas where houses may outnumber the breeding places but in most rural areas where the number of breeding places is astronomical this is a difficult approach (Davidson 1982). Then came the concept of shortening the lifespan of adult mosquito to prevent the completion of the extrinsic cycle of the malaria parasite. By spraying the resting places of female mosquitoes with the residual insecticides dramatic results were achieved in curtailing malaria transmission in early days of malaria eradication programme (WHO 1971). Unfortunately, with the appearance of resistance to hydrochlorine insecticides and the concern voiced by the environmentalists, the necessary switch to use less persistent and more expensive insecticide has been forced in several parts of the world. This also means that the future vector control must not solely rely on insecticides but should always be integrated with environmental, biological, educational and genetic methods.

Chemotherapy

Antimalarial drugs are employed both for curative and preventive purposes (WHO, 1990). The use of drugs for prevention of diseases or protection from malaria is referred to as chemoprophylaxis or chemoprevention. Antifolic drugs such as pyrimethamine and proguanil are essentially prophylactic drugs that prevent the maturation of the pre-erythrocytic forms developing in hepatocytes from the sporozoites. Primaquine is more lethal to pre-erythrocytic stages of malaria parasites. Most of the therapeutic antimalarial drugs used are curative or suppressive due to their blood schizontocidal properties. Quinine, a natural compound and chloroquine, a synthetic affordable drug are the two most popular schizontocides which had been given spectacular curative results. But in 1960 a new and threatening event occurred in the history of chemotherapy with the emergence of strains of *P. falciparum* resistant to the most widely used antimalarial therapeutic drug chloroquine (Maberti 1960). The menace of resistance to chloroquine is now widespread. It is a cause of concern as more and more strains of parasites from differ-
ent parts of the world are being reported to be resistant not only to chloroquine but also to some other antimalarial drugs (Peters 1989). Appearance of these polyresistant strains of malaria parasites has created an increasing clinical problem especially in treatment of *P. falciparum* in several parts of the world. At one time it was confidently presumed that the disease will be subjugated with accumulated weaponry against malaria, but experience has shown how sadly misplaced the judgement was. Today the existence of resistance in mosquitoes to insecticides and emergence of drug resistance in the parasites portray a grim scenario for future management unless new, effective tools are invented to combat the menace.

**OUTLINE OF THIS STUDY**

The search for antimalarial drugs, both natural and synthetic, has been and continues to be the most challenging and, at times, rewarding exercises ever undertaken by biologists and chemists. While most people engaged in the search for new drugs agree that a rational approach based on knowledge of the intimate biochemical pathways of the target cells would be ideal as well intellectually satisfying, most are reluctantly obliged to concede that up to present time, the chances of success following a more or less empirical search have been far greater. Spectacular advances in molecular biology and biochemistry in recent years, however, are rapidly changing this situation. New techniques for the study of the biology of malaria parasitism and for the cultivation of both intraerythrocytic and tissue stages of *Plasmodium* have opened up new avenues, not only for such fundamental studies but also for drug screening in vitro, the investigation of the modes of action of antimalarial drugs and the mechanisms of drug resistance. It is hoped, therefore, the future research on antimalarial chemotherapy will hinge more on intimate knowledge of the basic biology of the target organism and less on 'random' screening. In the present study basically we have evaluated erythrocytic schizontocidal properties of some natural and synthetic compounds, a series of
progressively resistant *P. falciparum* lines to dihydrofolate reductase (DHFR) inhibitors have been selected in vitro and mutation-specific diagnostic primers have been successfully used to discriminate the resistant and sensitive parasites by polymerase chain reaction (PCR).

In the present work a local *Plasmodium falciparum* isolate together with one of its fastest multiplying clones have been used for studies in vitro and for in vivo experiments a strain of *P. berghei* has been employed. **Chapter 1** contains a mini review on the antimalarial drugs, their known modes of action and mechanisms of drug resistance with emphasis on antifols and chloroquine.

The characterization of erythrocytic stages of *P. falciparum* parasites with regard to their susceptibility profile in vitro to some known natural antimalarial drugs (quinine and artemisinin) and commonly used synthetic drugs (chloroquine, amodiaquine, pyrimethamine and cycloguanil) against malaria has been described in **Chapter 2**. *P. falciparum* parasites have been found to be resistant to chloroquine and sensitive to all other natural and synthetic drugs evaluated.

In vitro relative chemosensitivity of erythrocytic stages of the chloroquine resistant *P. falciparum* isolate to quinine-type compounds has been given in **Chapter 3**. Apart from natural cinchona alkaloids, quinine and quinidine, their hydrogen derivatives proved to be more potent erythrocytic schizontocidal compounds. Some drug combinations show extremely good antimalarial property in vitro. Going by the IC$_{60}$ values of the eight preparations tested the compounds can be arranged in following order with respect to their schizontocidal activity:

quinidine/hydroquinidine ≥ hydroquinidine > quinine/hydroquinine ≥ quinidine > hydroquinine/quinine > apoquinine > epiquinine.

Clearly in depth investigations are required for exploiting the full antimalarial potentials of these cinchona derivatives or mixtures and other combinations.

Antiplasmodium properties of cisplatin (cis-platinum (II) diammine-dichloride), a neoplastic drug, have been assessed using in vivo and in vitro model systems of
malarial parasite (Chapter 4). A well tolerated dose of 6 mg/kg body weight of the compound cured the mice infected with *P. berghei* and the amount of cisplatin required for in vitro inhibition (IC₅₀) of a chloroquine resistant falciparum isolate was lower than either chloroquine or quinine. Minimum inhibitory concentration (MIC) needed to prevent the multiplication of asexual blood parasites in vitro was 30 ng/ml. Late ring and trophozoite stages of erythrocytic cycle were most susceptible whereas schizont and early ring stages were least sensitive to the toxic effect of cisplatin. Smaller multiple doses had been the most effective in curing malaria in mice than a single large dose. In a few cases, mice treated with a single intraperitoneal large dose of 6 mg/kg body weight, there was a delay in appearance of parasitemia but most of them recovered completely although slowly. This compound exerts its toxicity mainly by randomly damaging and cross-linking DNA strands as has been shown by Southern hybridization using a synthetic oligonucleotide probe, which is a repeat sequence in the falciparum genome. The report clearly demonstrates the antimalarial potentials of this compound and suggests a closer evaluation of this and other related compounds, specially in combination with antimalarial drugs to probe their synergistic properties.

Proguanil and pyrimethamine exhibit their antimalarial virtues by preferentially inhibiting the DHFR enzyme of parasites. Pyrimethamine has been deployed on a much larger scale than proguanil. These drugs have been extremely useful in treatment of chloroquine resistant falciparum infection but the limiting factor has been the quick development of resistance among the parasite population against these drugs. Pyrimethamine resistance is known to be widely distributed in all malaria endemic areas of the globe, including India. Proguanil has not been (extensively) used in India for over fifty years now. It should, therefore, be of interest to know the susceptibility of pyrimethamine resistant and sensitive lines of Indian origin to cycloguanil (the active metabolite of proguanil). These considerations prompted us to perform a series of experiments with a view to assess the
potential usefulness of cycloguanil in circumventing and combating the problem of pyrimethamine resistance in falciparum, which are presented in Chapter 5. It has been found that falciparum lines resistant to pyrimethamine are selected much faster than for cycloguanil resistance. Highly resistant pyrimethamine lines are predisposed for faster selection to cycloguanil resistance. Resistance acquired to pyrimethamine is stable. Pyrimethamine resistant parasites acquire a degree of cross resistance to cycloguanil and to another DHFR inhibitor, methotrexate but do not show any cross resistance to other groups of antimalarial drugs. Cycloguanil and pyrimethamine induce the formation of gametocytes in non-gametocyte forming clone. There has been no synergistic activity observed between cycloguanil and pyrimethamine. The deployment of triple drug combination of pyrimethamine, cycloguanil and sulfadoxine to impede the spread of resistance should be seriously considered.

Characterization of pyrimethamine resistant P. falciparum lines with a repetitive oligonucleotide probe and detection of pyrimethamine resistance using mutation-specific polymerase chain reaction has been presented in Chapter 6. In vitro test methods have widely been employed for determining the drug sensitivity of P. falciparum isolates but there are several limitations that affect their usefulness in early diagnosis of resistant falciparum infection. Resistance in P. falciparum to pyrimethamine and cycloguanil is known to be caused by point mutation(s) in the DHFR encoding sequence of the parasite genome. Feasibility of alternate assay methods for diagnosis of resistance to DHFR inhibitors based on genetic characterization and alterations has been explored. Restriction fragment length polymorphism (RFLP) and Southern hybridization with 21-oligomer probe, complementary to a repeat sequence existing in falciparum genome have been successfully applied to demonstrate the ability of this approach to detect prominent genetic alterations in the genome of highly resistant parasite DNA digested with Taq I or Alu I restriction enzymes. This probe shows that Hind III
restriction sites remain unaffected in pyrimethamine resistant lines. The RFLP studies clearly indicate that pyrimethamine causes point mutations in genome of the resistant falciparum parasites at places other than the DHFR encoding region. Low levels of resistance to pyrimethamine escape detection by this technique. This approach can be used to discriminate highly resistant and sensitive parasites but because of certain inherent shortcomings of Southern hybridization this method might not be employed for diagnosis of resistance. Polymerase chain reaction method has been explored to determine if mutation-specific primers can be used to diagnose pyrimethamine resistant parasites. Using the recent guidelines (Mullis 1994) for primer selection and consulting the falciparum DHFR gene sequence obtained from the databank (UNDP/World Bank/WHO/TDR), the selected primers have been optimized for the most stringent annealing temperature which could be employed for effective amplification and detection of pyrimethamine resistance. These initial precautions led us to discriminate the sensitive and resistant *P. falciparum* cell lines using the counter primer and the two diagnostic primers employed without compromising the sensitivity of the technique.