CHARACTERIZATION OF MALARIA PARASITES, IN VITRO SELECTION OF ANTIFOL RESISTANCE AND ITS DETECTION BY POLYMERASE CHAIN REACTION

THESIS SUBMITTED TO THE UNIVERSITY OF DELHI FOR THE DEGREE OF DOCTOR OF PHILOSOPHY DECEMBER, 1994

LATHIKA NAIR
DEPARTMENT OF ZOOLOGY UNIVERSITY OF DELHI DELHI - 110 007
CERTIFICATE

The work embodied in this thesis is original and has been conducted in the Department of Zoology, University of Delhi, Delhi. The work has not been submitted in part or full, to this or to any other University for any degree or diploma.

Date 14th Dec. '94

Lathika Nair

Head
Prof. R. N. Saxena
Department of Zoology
University of Delhi
Delhi - 110007

Supervisor
Dr. V. K. Bhasin
Department of Zoology
University of Delhi
Delhi - 110007
ACKNOWLEDGEMENTS

I take this opportunity to express my gratitude and sincere thanks to all those who have helped me during the tenure of my research work.

It is with deep sense of gratitude and indebtedness that I take this momentous opportunity to express my sincere regards to my supervisor Dr V.K. Bhasin for his , constant encouragement, support, fruitful scientific and academic discussions which often provided a new insight into the research problem. I am greatly obliged to him for his infinite patience, understanding, easy approachability, punctuality and unflinching moral support in face of odds which helped to finally shape this thesis.

I express my gratefulness to the Head, Department of Zoology, Delhi University and other faculty members in providing me with the necessary infrastructure and giving timely help and guidance.

I am much obliged to Dr Fred Kironde of ICGEB (International Centre for Genetic Engineering and Biotechnology) for his magnanimity in providing help whenever required.

I fall short of suitable words to express my deep sense of gratitude to my parents for their love and affection, understanding, endless patience, inspiration, encouragement and moral support. I wouldn't have been successful in completing this thesis without their blessings.

The constant encouragement and cooperation all along of Ms. Neera Mehra is thankfully acknowledged.

My sincere thanks are due to my laboratory colleagues Ms. Swaty Nigam, Ms. Akhila Aggarwal and Mr. Vikas Goel for their unstinted support and assistance specially during the final compilation of this thesis.

I express my warm appreciation to Mr. Shashi Bhushan for the assistance rendered in laboratory work. I sincerely appreciate the help given by Mr. E.A. Daniel and Mr. Ukil for the photographic work. My thanks are due to Mr. R. Bhandari for his assistance.

Fellowship from University Grants Commission, Government of India, is duly acknowledged.
CHARACTERIZATION OF MALARIA PARASITES, IN VITRO SELECTION OF ANTIFOL RESISTANCE AND ITS DETECTION BY POLYMERASE CHAIN REACTION

GENERAL INTRODUCTION

| Introduction to malaria | 1-9 |
| Outline of this study   | 9-13 |

SECTION - I

| Chapter 1 | Antimalarial drugs, their known mode of actions and mechanisms of drug resistance - a brief review | 14-33 |
| Chapter 2 | Susceptibility of *Plasmodium falciparum* isolate and a clone to a few antimalarial drugs in vitro | 34-52 |
| Chapter 3 | Chemosensitivity of a chloroquine-resistant *P. falciparum* isolate to quinine-type compounds in vitro | 53-60 |
| Chapter 4 | Cure with cisplatin of murine malaria infection and inhibition in vitro of a chloroquine resistant *P. falciparum* isolate | 61-72 |

SECTION - II

| Chapter 5 | In vitro selection of *P. falciparum* resistant lines to dihydrofolate-reductase inhibitors, cross resistance and other related studies | 73-94 |
| Chapter 6 | Characterization of pyrimethamine resistant *P. falciparum* lines with a repetitive oligonucleotide probe and detection of resistance using mutation-specific polymerase chain reaction | 95-117 |

REFERENCES | 118-140 |
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:

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Apicomplexa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class</td>
<td>Sporozoa</td>
</tr>
<tr>
<td>Subclass</td>
<td>Coccidia</td>
</tr>
<tr>
<td>Order</td>
<td>Haemosporida</td>
</tr>
<tr>
<td>Suborder</td>
<td>Aconoidina</td>
</tr>
<tr>
<td>Family</td>
<td>Haemosporidae</td>
</tr>
<tr>
<td>Genus</td>
<td><em>Plasmodium</em></td>
</tr>
</tbody>
</table>
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 oo-
cysts. Meanwhile reduction division has occurred following recombination and
haploid genome is restored. The oocyst undergoes repeated mitotic divisions fol-
lowed 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 surdardes* and was named *Plas-
modium 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 malarias, *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, *P. vivax* and *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. *P. malariae* is also restricted to a subpopulation of erythrocytes, probably older ones (Pasvol and Wilson 1982). Another distinctive feature of *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 μl of blood (Bruce-Chwatt and Zuluera 1980). Even 20 to 40 parasites μl of the blood can be detected (on an average 1 μl 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 in spite 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
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$_{50}$ 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 (IC$_{50}$) 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.
Figure 1 Diagrammatic sketch of the life cycle of human (P. falciparum) and rodent (P. berghei) malaria parasites.

1. Mosquito
2. Feeding | Stomach (midgut) | Salivary glands
3. Exflagellation
4. Macrogametocyte
5. Microgametocyte
6. Male gametocyte
7. Female gametocyte
8. Female mosquito with sporozoites
9. Fertilization
10. Oocyst formation
11. Oocyst within midgut wall
12. Mature oocyst with sporozoites
13. Parasite within liver parenchyma cell
14. Exoerythrocytic schizogony
15. Erythrocytic schizogony
16. Schizont
17. Mature RBC
18. Hepatocytoma
19. Erythrocytic development
20. Gametocytes
21. Rodent/Human
22. Human
23. Rodent
24. Rodent
25. Mosquito
26. Host
27. Blood stream
Section - I
Chapter 1

ANTIMALARIAL DRUGS, THEIR KNOWN MODE OF ACTIONS AND MECHANISMS OF DRUG RESISTANCE - A BRIEF REVIEW

Abstract: Brief historical review of antimalarial drug development and deployment shows that en masse drug deployment for chemoprophylaxis and monotherapy inevitably results in quick selection of drug resistance in Plasmodia. Drug combinations survive longer. The chemical structure, biological activity and mode of action of important antimalarials of the century have been discussed. Mechanisms by which parasites become resistant to dihydrofolate reductase (DHFR) inhibitors have been identified to specific point mutations in the DHFR gene of the parasite, thus altering the structure of the enzyme which avoids affinity binding of the drug. Advent of resistance to chloroquine, the most affordable low cost drug, is a cause of concern. Our inadequate knowledge about the subject is updated and highlighted for inclusion in future research agenda among other listed priorities. Old remedies like 'qinghaosu', quinine and other cinchona alkaloids have brought new hopes for the time being.

INTRODUCTION

Around the turn of this century, malariology enjoyed certain vogue, inspired largely by: a) the interest of colonial powers in places where malaria was rampant; b) the explorative expeditions to the tropical locations of the developing world; and c) the exciting discoveries of Ross, Ehrlich and others. The popularity and excitement in the study of malaria subsided for next few decades in favour of prokaryotic infectious diseases. Short supply of the main antimalarial drug of the time, quinine, was experienced during the two World Wars by the developed countries. This shortage in the meanwhile had instigated the efforts in malaria research leading, immediately after the Second World War, to two dazzling events which were to determine and dominate malarial strategy for next few decades. The first was the success of the residual insecticides in dramatically curtailing transmission of malaria in many countries and the second was the development of more than one synthetic antimalarial drugs which became available in rapid succession. Never before had man stockpiled such powerful arsenals to rid himself of malaria malady, so old an adversary. This led enthusiastically in 1955 to launching of malaria eradication programmes globally by the World Health Organization (WHO), one of the largest international efforts to improve the health in the developing world, but the initial enthusiasm slowly faded due to several technical as
well as socio-economic factors and this venture finally ended in 1970 when WHO decided to 'throw in the towel'. During this period support for further research into malaria had dried up throughout the world. In the meantime casualties inflicted to the U.S. forces in Vietnam War by drug-resistant parasites prompted the developed world to know more about the cure and prevention of malaria. This not only revived but intensified research and funding into several aspects of biology of parasitism of Plasmodium (causative agent of malaria) including molecular biology, immunology, vaccinology and chemotherapy. The sudden surge of research activity on plasmodia demanded ever increasing supplies of the parasite material, infecting man in particular, thus stressing an urgent need to tame the parasites in vitro. In 1976 Professor William Trager reported (Trager and Jensen 1976) the first successful continuous cultivation of erythrocytic stages of P. falciparum (causing malignant tertian human malaria) and this salutary breakthrough stimulated further research activity related to malaria parasites. It also provided a fresh impetus to monitoring of the emergence and spread of drug resistance in falciparum throughout the world. This article discusses the basic structure and biological activity of important antimalarial drugs used in the current century with special emphasis to chloroquine and DHFR inhibitors, their modes of action and mechanisms of resistance, analysis the causes leading to speedy selection of resistant parasites, suggests measures to impede the spread of resistance and future research strategies for development of new antimalarial drugs.

**Basic Chemical Structure of Antimalarials**

Known antimalarials have been variously grouped. They may be classified based on the following basic chemical structures (Figure 1.1):

*Pyrimidine derivatives*: The amino groups at the 2 and 4 positions of the pyrimidine forms the basis of synthetic antimalarials like pyrimethamine and other less popular compounds.

*Quinoline derivatives*: Fusion of a pyridine and a benzene ring gives rise to a
bicyclic quinoline ring. It forms the basis of a number of synthetic antimalarial compounds which are derivatives of the 4 or the 8 amino substituted quinoline. Chloroquine, a 4-aminoquinoline and primaquine, a 8-aminoquinoline are the two well known examples. Some of the natural cinchona alkaloids also have quinoline in their structure, quinine for instance is formed by complex substitution at the 4 position of the quinoline ring.

**Acridine derivatives**: Acridine is a polycyclic compound formed by the fusion of two benzene rings and pyridine. Mepacrine is an antimalarial drug which is a 9-amino acridine derivative belonging to this group. It is largely obsolete as an antimalarial for causing coloration of the skin and transient mental disturbances.

**Non-cyclic derivatives**: A few groups of antimalarials posses non-cyclic basic structure and substitution at some positions with benzene ring derivatives. They are the biguanide and the sulfa compounds. The sulfa compounds may further be grouped into sulfones and sulfonamides. Proguanil is an antimalarial drug derived from biguanide. Sulfadoxine and dapsone are the derivatives obtained from the modification of sulfonamide and sulfone, respectively.

**Basic Biological Activity of Antimalarials**

Antimalarial drugs can also be classified based on their predominant site of action on the particular stage(s) in the life cycle of malarial parasite. There are four species of *Plasmodium* (*P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*) usually known to infect man. Malaria results when the infective stages of the parasite are injected into the bloodstream of man by an infected female *Anopheles* mosquito. The sporozoites immediately take shelter within hepatocytes of the host for further development. Maturation of all these intracellular protozoan parasites finally results in the release of pre-erythrocytic merozoites, almost simultaneously in *P. falciparum* and *P. malariae*, whereas in *P. vivax* and *P. ovale* some of the liver stage parasites may remain dormant for variable duration, for
months or years. These dormant stages are called hypnozoites, subsequently blossoming into pre-erythrocytic merozoites, causing relapse of malaria. The drugs which prevent the maturation of pre-erythrocytic stages are called tissue schizonticides and those acting on the hypnozoites are known as anti-relapse drugs. The pre-erythrocytic merozoites invade the red cells leading to the formation of daughter merozoites, by schizogony (asexual division), which on release invade the uninfected erythrocytes. The pathogenicity of malaria is chiefly due to synchronized burst of erythrocytic schizonts leading to malaria symptoms, namely fever, shivering, hemolytic anemia and sometimes (in infection with P. falciparum) to cerebral malaria and death. Blood schizontocides are toxic or lethal to intraerythrocytic parasites and thus relieve the host of malaria symptoms. Some of the merozoites on entering the erythrocytes start differentiating into the gametocytes (sexual forms) instead of undergoing usual asexual multiplication. The mature gametocytes will develop further only in the female Anopheles. The drugs which prevent the formation or differentiation of sexual forms are termed as gametocytocidal drugs. Male and female gametocytes form respective gametes which fertilize in the stomach of the insect vector to form the diploid zygote. The zygote continues its sporogonic development from oocyst to sporozoites. The compounds interfering with the sporogonic cycle are recognized as sporontocidal compounds, thus preventing the transmission of malaria. They have also been called anti-sporogetic chemicals.

Mechanisms of Action and Resistance

The main technical reasons for the failure of malaria eradication programme had been the development of resistance (exophilly) to residual insecticides by insect vectors and the emergence of drug resistance in parasites to the antimalarials. Improvements in malaria chemotherapy should result from better understanding of the mechanisms that govern drug action and drug resistance. The two criterions of chemical structure and biological activity of antimalarials on plasmo-
dia have been clubbed for convenience to discuss some of the known modes of action and resistance to antimalarial drugs.

1. Folate Pathway Blockers

A Mode of Action: Folate pathway blockers includes the antimetabolite drugs which interfere with folic acid cycle of the parasite, in one way or the other. Reduced folic acid derivatives are indispensable cofactors for de novo synthesis of pyrimidines in plasmodia. In the absence of any salvage pathway for the utilization of exogenous pyrimidines, depletion in the pool of reduced folic acid derivatives in the parasite milieu results in blocking of Plasmodium multiplication. The folate pathway blocker antimalarial drugs can be grouped into two broad categories, the dihydrofolate reductase (DHFR) inhibitors and the sulfa drugs which are structurally similar to para-aminobenzoic acid (PABA). Two commonly used DHFR inhibitor antimalarial drugs are pyrimethamine and proguanil whereas sulfonamides and sulfones are employed as antimalarials mostly in combination with pyrimethamine.

Proguanil was developed in Britain during the Second World War. While evaluating derivatives of pyrimidine, compounds of the chloroguanil series proved promising, opening the pyrimidine ring further simplified the structure to biguanide (Curd et al. 1945), which were to have good antiplasmodial activity. Thus proguanil (chloroguanide) was discovered. The efforts of British group in collaboration with American scientists further culminated in the development of pyrimethamine (Falco et al. 1951), first introduced in 1952 for routine human use. Both the drugs are employed for prophylaxis and suppression of malaria infection in man. They act on the dividing stages of the parasites but not on the dormant (hypnozoite) or growing sexual stages (developing gametocytes). They have the important properties of low toxicity. Pyrimethamine has added advantages of being effective in much smaller doses and longer half life with slow excretion rate compared to proguanil.
Proguanil and pyrimethamine act as antimalarial drugs by virtue of their great affinity for the plasmodial enzyme DHFR (Ferone et al. 1969). The enzyme gets inhibited following binding of the drug. DHFR catalyzes the conversion of dihydrofolate to tetrahydrofolate and occupies a key position in cellular biosynthesis of purines, pyrimidines and certain amino acids. DHFR is present both in the parasite and the host cells. The basis of selective action of these drugs is their differential (greater) binding to the plasmodial DHFR and the extraordinary sensitivity of this action to the nuclear division of the malaria parasite at the time of development of schizonts in erythrocytes and in the liver tissue (Schellenberg and Coatney 1967; Gutteridge and Trigg 1971; Inselburg and Banyal 1984; Janse et al. 1986). The affinity of *P. berghei* (rodent malaria parasite) DHFR for pyrimethamine is 2000 fold greater than the mouse erythrocyte enzyme (Ferone et al. 1969). Proguanil is not active against plasmodia in vitro but one of its metabolite, cycloguanil is the active intermediate having chemical structure somewhat similar to that of pyrimethamine (Carrington et al. 1951) (Figure 1.1). The activation of proguanil to cycloguanil is done by the mixed function oxidase system of the hepatic microsomes (Armstrong and Smith 1974). It is not known if pyrimethamine and cycloguanil bind to the same region of the plasmodial DHFR or occupy different sites.

Spectacular successes of sulfonamides as antibacterial drugs in 1930s led to the evaluation of their antiplasmodial action. In 1937 the antiplasmodial activity of some of the derivatives was recognized against human malaria (Anand 1979). The sulfonamides and sulfones exert activity against all actively dividing stages in the life cycle of plasmodia. Among the human plasmodia *P. falciparum* is more susceptible, whereas *P. vivax* is less so (Bruce-Chwatt et al. 1981). The earlier developed sulfa drugs acted slowly for short duration and the need for high and potentially toxic doses were responsible for their shelving, as also more reliable 4-aminoquinoline compounds became available. However, advent of resistance to 4-aminoquinolines has revived interest in sulfa drugs as they retain full activity
against chloroquine resistant parasites (Peters 1990a) and these drugs act synergistically with the DHFR inhibitors (Bartelloni et al. 1967; Harinasuta et al. 1967). The mode of action of the sulfonamides and sulfones against plasmodia is considered analogous. Antimalarial sulfa compounds are structurally similar to p-aminobenzoic acid and the commonly accepted view is that these drugs are competitively antagonized by PABA and non-competitively by folic acid (Watkins et al. 1985). PABA is incorporated into the coenzyme folic acid; inhibition of folic acid synthesis by these drugs has been demonstrated and the enzyme involved was to be tetrahydropteric acid synthetase. Another study supports the view that altered permeability of the erythrocyte membrane might be responsible for action of sulfa drugs as dapsone has been demonstrated to accumulate at the surface of rat erythrocytes (Canedella and Jarrell 1970), leading to the reduced uptake of glucose in the erythrocytes and inhibition of glucose consumption by intraerythrocytic parasite.

B. Mechanism of Resistance: The use of DHFR inhibitors as antimalarials has been curtailed because of rapid development of resistance by the parasites. Shortly after the release of each drug, prophylactic and therapeutic failures against Plasmodium infections were reported (Peters 1970) from various places. Resistance to DHFR inhibitors by several strains of Plasmodium has subsequently become a major problem throughout the world. The ease with which resistance to DHFR inhibitors developed both in the field and laboratory suggested that single mutation in the Plasmodium DHFR segment of the genome might be enough to render these drugs ineffective (Bishop 1962; Diggens et al. 1970; Peters 1987). The experimental evidence as to the genetic basis of resistance came from the analysis of a cross between pyrimethamine resistant and sensitive lines of rodent malaria resulting in inheritance patterns compatible with the involvement of just a single gene locus (Walliker et al 1975, 1976; Knowlesi et al. 1981). Kinetic studies further revealed that DHFR from resistant Plasmodium binds less tightly to py-
rimethamine, as there was marked increase in inhibition constant (K<sub>i</sub>) with respect to this drug (Ferone 1970; Sirawaraporn and Yuthavong 1984; Mc Cutchan et al. 1984; Walter 1986; Dieckman and Jung 1986), strongly suggesting the altered structure of the enzyme DHFR in resistant parasites. An important feature of <i>P. falciparum</i> DHFR is that the activity resides within a bifunctional protein together with thymidine-late synthetase (TS) (Garrett et al. 1984). Another novel property of the DHFR from resistant parasites is that it retains its normal enzymatic function but at the same time inhibits binding of the drug. First complete sequence of DHFR-TS was published in 1987 (Bzik et al. 1987). There work showed that as in <i>Leishmania</i> there is a single intronless open reading frame that includes a junction region linking the DHFR and TS domains. The DHFR domain extends from the N-terminus to amino acid residue 225 approximately, while the TS domain occupies roughly the final 290 amino acids of the protein leading to the C-terminus at residue 607. The junction regions thus extends from about residue 225 to residue 320. Many of the or postulated active site residues in both DHFR and TS are found to be conserved in the <i>P. falciparum</i> molecule (Bzik et al. 1987; Cowman et al. 1988). The DHFR-TS gene has been shown to reside on the fourth fastest chromosome in several strains (Peterson et al. 1988; Cowman et al. 1988; Snewin et al. 1989; Tanaka et al. 1990). Ever since, structure of the DHFR gene from number of resistant and sensitive falciparum clones/isolates have been compared by sequencing. This rapid accumulation of sequencing data has been made possible by in vitro amplification of DHFR region of the genome using polymerase chain reaction (PCR). Analysis of sequence data of natural resistance in diverse number of geographical distinct isolates shows that a single point mutation (resulting in Ser. 108 to Asn. 108) in the DHFR active site is perfectly linked to pyrimethamine resistance (Table 1.1). Two ancillary mutations Asn. 51 to Ile. 51 and Cys. 59 to Arg. 59 were found to be associated with increased level of resistance in association with Asn. 108 (Cowman et al. 1988; Peterson et al. 1988; Snewin et al. 1989; Tanaka et al.
Experimentally induced resistance in *P. falciparum* using mutagens in vitro (Banyal and Inselburg 1986) showed that amino acid changes at entirely different sites than that seen in naturally resistant pyrimethamine parasites (Inselburg et al. 1988; Tanaka et al. 1990). This clearly shows that there are many sites in the DHFR gene where point mutations can alter the structure of the enzyme, preventing or retarding drug binding, without compromising with catalytic functions. More studies are needed to ascertain the extent of polymorphism of these sites in diverse geographical isolates.

The finding that specific changes in DHFR base pairs are linked to pyrimethamine resistance indicated that point mutations might also be responsible for resistance to cycloguanil. Sequence data analysis of cycloguanil resistant DHFR revealed that parasites with paired Ser. 108 to Thr. 108 and Ala. 16 to Val. 16 mutations were found to be resistant to cycloguanil but showed only a slight decrease in sensitivity to pyrimethamine. Parasites refractory to both the DHFR inhibitors had three mutations in common Cys. 59 to Arg. 59, Ser. 108 to Asn. 108 and Ile. 164 to Leu. 164 (Foote et al 1990; Peterson et al 1990). Identification of specific mutations in DHFR segment of genome opens up the possibility of mutation specific oligonucleotide probes, using PCR, capable of differentiating sensitive and resistant parasites (Zolg et al, 1990).

Resistance to sulfas has not been widely recorded with human plasmodia. For many years these drugs were exclusively used in combination with DHFR inhibitors mainly pyrimethamine. Of late there have been reports of emergence of resistance to these combinations from Brazil and Southeast Asia in *P. falciparum* (Brockelman and Tan Ariya 1982). There is, of course, little doubt that plasmodia resistant to the combination also are resistant to sulfas alone.

### 2. Quinoline-Containing Antimalarials (QCA)

They essentially include blood schizonticides (except for primaquine). Cinchona alkaloids (quinine) and other 4-
quinolinemethanol (mefloquine); chloroquine (4-aminoquinoline), primaquine (8-aminoquinoline), are some of the drugs dealt in this section.

**Cinchona Alkaloids:**

Quinine, one of the major alkaloid components of cinchona bark, has saved mankind from ravages of dreaded malaria disease for more than 350 years and continues to cure malaria, any malaria even today! It acts on the multiplying asexual stages of the parasite but has no lethal effect on the exoerythrocytic stages, it is gametocytocidal for *P. vivax* and *P. malariae* but toxic only to the developing gametocytes of *P. falciparum*. Quinine toxicity to malaria parasite is based on concentrative uptake of the drug in infected erythrocytes leading to cessation of motility and haemozoin formation. It stands apart, in an era, when life cycles (usefulness) of drugs are mostly expressed in decades rather than centuries. Indiscriminate use of this drug shall definitely lead to selection of resistant strains of *Plasmodium*, as had been the case in the past with other drugs. We cannot afford this to happen now, at the time when quinine seems to be the only effective drug against the multi-drug-resistant (polyresistant) malaria strains. Time demands the strategies to prolong and preserve the antimalarial efficacy of cinchona alkaloids and derivatives. The question is how and why this drug has survived as an effective antimalarial for so long. If that is answered then perhaps one might be able to suggest ways to delay the emergence of resistance to this drug. Mostly crude bark was used for preparation of powder and infusion. As we know now that this extract from 'bark to bottle' contained not only quinine but other alkaloids as well in varying amounts, some of them even more potent than quinine. Even the 'pure' quinine salts, as quinine sulphate, available contained other cinchona alkaloids (Smit,1987) to the extent of 16% in 1890, 8.5% in 1970 and 6% in 1986 (Figure 1.2). Clearly the alkaloid mixture used in the past for treatment, survived in retaining the antiplasmodium virtues for centuries. Even modern medicine
advocates drug combination over monotherapy to prolong the life span of the drugs (Peters 1990c). Quinine is not the most effective of the four major alkaloids found in cinchona bark. In vitro also some of the cinchona alkaloids and synthetic derivatives are more potent than quinine (Nair and Bhasin 1993). It has reached its unique position because it just happened to be the first to be isolated and used in 'pure' form. Perhaps the solution to the malaria problem even today can be rediscovered where it was first found - in the bark of cinchona tree. Of late, increasing failure rates of old and new synthetic drugs has again drawn the attention of research workers to the oldest natural antimalarial drug.

*Mefloquine (4-quinolinemethanol):*

Mefloquine may be considered the third generation quinolinemethanols. The first generation is represented by cinchona alkaloids, the second (less known) by the compounds in which the cinchona side chain was modified with introduction of 2-phenyl substituent and the third by mefloquine in which the 2-phenyl moiety is replaced by a trifluromethyl group (Figure 1.1.). Each generation of quinolinemethanols brought an improvement in the therapeutic properties of the representative drugs. Mefloquine is well-tolerated, effective in single dose against *P. falciparum*, as its half life is for about 14 days in man, with cure rate of 100%. It can be used as suppressive prophylactic drug for both vivax and falciparum malaria. It does not have tissue schizonticidal activity and thus cannot produce radical cure with relapsing malaria infections. It was first synthesized in 1971 and improved method of synthesis patented in 1978 (Sweeny, 1984). Parasites become quickly resistant to the drug specially chloroquine resistant ones. Thus it has a limited utility for mass use as a single drug for malaria treatment. However, it can be beneficially employed in drug combinations with caution.

*Chloroquine and other 4-aminoquinolines:*
Presence of quinoline ring in the two antimalarials, quinine and pamaquine, was known to the Germans by 1930. This prompted them also to explore several derivatives of 4-aminoquinolines, resulting in the final selection of chloroquine and sonoataquine for further trials in North Africa. Sontaquine was preferred over chloroquine by the Germans for being less toxic. Supplies of this drug together with German research data were captured by United States forces in Tunis which stimulated further studies in USA on these and other 4-aminoquinolines (Coatney, 1963; Peters, 1987). Chloroquine was found by US workers to be the most effective among them and less toxic on volunteers than any other. So the drug which was synthesized in 1934 in Germany was rediscovered in USA in 1946. Chloroquine and other 4-aminoquinolines have been found to be highly effective against the asexual blood stages of all four species of malaria (prior to appearance of resistant strains). They produce rapid clinical cure and are excellent suppressive agents. Parasitemia disappears within 48-72 hours following standard therapeutic regimen. They are gametocytocidal agents against all human malaria parasites except mature \textit{P. falciparum} gametocytes. Since release this inexpensive drug has been widely used both for treatment and prophylaxis. Its consumption had been progressively increasing for instance 265,052 kg base was actually consumed in 1978 and the demand rose to estimated 351,229 kg base in 1985, representing respectively 177 million and 234 million adult therapeutic doses (WHO, 1984). By far the most enormously consumed synthetic antimalarial drug in the history of mankind. Naturally, therefore, the most extensively studied antimalarial at the same time surprisingly our knowledge about the mode of action of this drug is far from complete. One wonders if this deplorable situation is due to complexity of the subject matter or absence of appropriate technology to resolve the knots or simply lack of more inquisitive minds to join the endeavour to unfold the specific mechanism of drug action. Whatever might be the case, it calls for periodical update about this riddle. If we knew the exact mode of action then per-
haps one could tackle more rationally the evolution of resistance to these drugs in the parasites and design effective strategies to combat or contain the ever increasing menace of resistance to chloroquine. We will restrict our deliberation to some of the recent discoveries in this field as reviewed by Ginsburg and Krugliak (Ginsburg and krugliak, 1992).

**Concentration of QCA in Parasitized Erythrocytes:**

QCA have variety of biological effects on plasmodia depending upon their concentrations. Chloroquine concentration of $10^{-7}$ M, for instance, is sufficient to bind ferriprotoporphyrin IX (FP) and form toxic complex; concentration of $10^{-6}$ M or greater inhibits lysosomal functions and concentration of $10^{-4}$M or greater permit drug binding to the major cellular constituents and, presumably, prevent their functioning (Mc Chesney et al 1984). Most of the discussion on QCA relates to chloroquine.

**Inhibition of Lysosomal Functions:**

QCA are amphiphillic weak bases and driven freely across the membranes towards the acid digestive vacuoles of the malaria infected erythrocytes (Aikawa 1972). Extent of accumulation of the drug is dependent on the pH gradient of the lysosomal vacuole and the extracellular medium (Yayon et al. 1984). Once inside the acid vacuoles the QCA bases become protonated and their free permeability across the membranes is reduced by several folds (De Duve et al. 1974). The protonation of the influxed bases in the lysosomal compartment causes temporary alkalinization and is counteracted by the vacuolar proton pump (Geary et al. 1986). Alkalinization inhibits metabolism in the lysosomes and decline in the pH gradient decreases the drug influx. It had, therefore, been postulated that drug accumulation causes increase in the vacuolar pH resulting in inhibition of the vacuolar enzymes. Protease inhibitor, leupeptin, causes reversible cessation of digestion in the parasite whereas impedance of digestive process due to QCA is nonre-
versible (Rosenthal et al, 1988). But there is insignificant change in the pH of lysosomal vacuoles at the pharmacological level of QCA to cause any inhibition of lysosomal functions, as has been demonstrated (Ginsburg et al, 1989).

**Interaction of QCA with Ferriprotoporphyrin IX :**

Intraerythrocytic parasite consumes internalized hemoglobin by proteolysis in the lysosomes. Toxic residue FP or oxidized heme so formed is polymerized by the parasite into a crystalline nontoxic substance called haemozoin or malaria pigment. It has been proposed that chloroquine interferes with the formation of nontoxic compound by complexing with FP, thus lethally poisoning the parasite (Fitch 1983 ; Fitch 1986). These complexes not only contribute to inhibition of digestion through their effect on the parasite's acid cysteine protease but also can disrupt integrity of vesicle membranes resulting in lysis (Orjih et al. 1981; Fitch et al. 1982). However, QCA treated parasitized cells do not show either morphological or biochemical disruption of the vacuolar membranes which corroborates the finding that lytic effect of the complexes is prevented by the serum proteins (Zhang and Hempelmann, 1987). Also the failure to demonstrate presence of QCA in the necessary amounts are incompatible with this suggestion. This does not rule out the possible role of QCA-FP complexes in the antimalarial action. In fact, since they can be formed only in malaria-infected cells, they are good candidates for accounting for the specific antimalarial actions. Inhibition of heme polymerase by QCA has been recently implicated as mode of action (Slater and Cerami, 1992), also QCA preventing the release of iron from the acidified host cell cytosol, a possible source of this trace element for the parasite has also been proposed (Ginsburg and Krugliak, 1992).

**Intercalation of QCA with DNA :**

Chloroquine, quinine but not mefloquine have been found to inhibit DNA
synthesis by intercalating with DNA but at concentrations four orders above the pharmacological levels of the drugs. Another serious flaw with this hypothesis is that chloroquine has higher affinity to GC nucleotides (Cohen and Yielding, 1965) but plasmodia DNAs are AT rich (Weber, 1988), it is difficult to account for selective action of chloroquine against plasmodia.

Mechanisms of Chloroquine Resistance:

Advent of chloroquine resistance is one of the principal factors for the present global resurgence of malaria. Resistance in the parasites may develop either due to reduced accumulation of the drug or because of alterations in the drug target-site of the parasite. Drug target has not yet been identified but there is ample evidence that resistant strains of *P. falciparum* accumulate less chloroquine (Krogstad and Schlesinger, 1987). Decreased activity of vacuolar proton pump (Ginsburg and Krugliak, 1989) has been implicated in reduced accumulation of the drug in lysosomes and not due to the activity of any efflux pump. Acidotropic chloroquine is driven to lysosomes by pH gradient maintained by hydrogen pumping into the vacuole, slowing of the pump activity causes lowering of pH gradient which in turn leads to reduced concentration of the drug in resistant parasites. Drug resistance is associated with amplification of multiple drug-resistant (*mdr*) genes and their products in cancer cells (Endicott and Ling, 1989). By analogy with this situation two *mdr* genes have been identified in *P. falciparum* (Wilson et al., 1989) and one of them *Pfmdr 1* is markedly homologous to human *mdr* gene (Foote et al., 1989). In cancer cells *mdr* gene products are involved in efflux of the accumulated drug but in malaria the existence of similar *mdr* vacuolar pumps seems doubtful at present, *mdr* products might be involved in imparting resistance by some other mechanism, also amplification of these genes have not been found in all resistant falciparum strains. Sequencing of *Pfmdr1* gene showed that resistant isolates differ by one to four nucleotides from chloroquine sensitive genes.
in some strains but were same in others (Foote et al. 1990).

**Resistance Reversers:**

Compounds like verapamil (Martin et al 1987), diltiazem, vinblastine (Ginsburg and Krugliak, 1992) etc. are known to reverse drug resistance in cancer cells. They are similarly effective in reversing chloroquine resistance in resistant falciparum, mostly in vitro. A psychotropic agent, desipramine, is effective in reversing falciparum resistance at well tolerated dose in *Aotus* monkeys (Peters 1990a). Similarly cyproheptadine and several other related antihistaminic agents have shown the ability to reverse chloroquine resistance against *P. falciparum* both in vitro and in vivo. However, clinical applications against malaria is still far from infancy.

**Primaquine (8-aminoquinoline):**

Most widely used tissue schizontocide and antirelapse drug (for vivax and ovale infections) since its introduction in 1940s. Its gametocytocidal and sporontocidal virtues are important operationally, since a single dose might reduce the transmission of drug-sensitive and resistant falciparum malaria. Clinically ineffective against asexual blood stages. Although primaquine is the safest available 8-aminoquinoline it does have significant toxic effect on individuals with glucose-6-phosphate dehydrogenase deficiency. Primaquine inhibits parasite mitochondrial respiration and this is probably the basis of its action. Not enough is known about its exact mode of action and resistance status to this drug.

3. **Artemisinin:** *Artemisia annua* a herb of family Compositae has been used in malaria therapy in China for over 1000 years but its active antimalarial component, artemisinin or 'qinghaosu' was not isolated and characterized until 1972 by the Chinese scientists. Artemisinin and a derivative, artemether, are capable of curing patients with severe malaria resistant to most other drugs. Artemether
has been found to be more effective in preventing deaths from malaria than intravenous quinine - the traditional treatment for severe malaria. It is safe and fast acting blood schizonticide, a potential life-saving advantage in severely ill patients. Some of its derivatives have recently been shown to possess better schizontocidal and gametocytocidal properties in vitro (Mehra and Bhasin, 1993). Artemisinin has the structure of sesquiterpene lactone with an internal peroxide linkage. The peroxide moiety appears to be indispensable for chemotherapeutic activity. The definitive mode of action of this series of drugs is still not known and resistance to these compounds can be produced in vitro (Inselburg 1985). No doubt this old remedy brings new hope for the time being if deployed judiciously.

**Factors contributing to the selection of drug resistant parasites**

In the following section some of the factors which are likely to play an important role in the emergence and propagation of drug resistance in malarial parasite are considered.

*Mass drug administration:* Most forms of mass drug administration employ the medicament at subcurative doses, thus leading to the selection of less sensitive or resistant parasites. This is of limited importance while malaria transmission is interrupted or reduced to a very low level (Wernsdorfer and Kouznetsov 1980). However, if drug pressure is exerted whilst transmission is intensive and unabated, selection and subsequent propagation will be rapidly effected. This effect is most marked under conditions of universal drug pressure as is exerted by the use of medicated salt (Verdrager 1986 a,b).

*Inadequate treatment:* A strong correlation between the immune status and drug response in areas with intensive malaria transmission has been observed especially in children of different age groups. For many years chloroquine was administered as a single dose of 10 mg/kg body weight for providing radical cure in semi-immune subjects for instance in tropical Africa (WHO 1986). When infants and children not sufficiently immune were treated with the same dose the drug failed
to provide radical cure. The dose was thus subcurative and therefore conducive to the selection of resistant parasites (Gbarry et al. 1988; Ramanamirija et al. 1985).

**Vectorial factors:** Studies in Central and West Africa indicate that the risk of contracting chloroquine resistant *P.falciparum* is directly correlated to vector density and sporozoite rate (Brandicourt and Gentilini 1987). The propagation of chloroquine resistant *P. falciparum* was quite slow in the areas of moderate and low transmission where sensitive *P. falciparum* continues to prevail in spite of an early onset of chloroquine resistance in the hyper-endemic lowland regions of Madagascar (Le-Bras et al. 1987).

**Population mobility:** Migration has been identified as a major contributory factor to the occurrence and spread of drug resistance (Verdrager 1986 a,b). Migration is an important determinant in the transportation of the infection to unaffected areas. Intensity of migration in association with a different intensity of drug use may also cause major differences in the prevalence of resistant infections within relatively small geographic areas as was shown in the example of two population strata in Amazonian Brazil (Kremsner et al. 1989).

**Biological advantage of resistant parasites over sensitive ones:** Chloroquine resistant *P. falciparum* differs in certain biological characteristics from chloroquine sensitive populations. It has proved to be more infective to *Anopheles stephensi* and also to produce large number of oocysts: an effect also observed in *A. dirus* (Sucharit et al. 1977). Chloroquine may enhance the infectivity of chloroquine resistant plasmodia to anophelines. This has been shown in *P. berghei / A. stephensi* model (Ramkaran and Peters 1969). A marked decrease in sensitivity to chloroquine has been observed in *P. falciparum* isolates after continuous growth for long periods in vitro culture in the absence of drug pressure (Le Bras et al. 1980).

**Lessons from Past:**

History of drug resistance shows that whenever a new antimalarial was deployed *en masse* for monotherapy and chemoprophylaxis resistance to the drug
Figure 1.1 Chemical structures of antimalarial drugs
appeared quickly. Life span of drug combinations had been longer. Misuse of drugs has also contributed to the fast selection of resistant parasites, which should be prevented as far as possible. Antimalarials should only be deployed as one of the tools in an integrated malaria control programme. 'Think globally and act locally' should be the guiding principle for any malaria control programme.

**Future Agenda:**

The current grim situation is a cause of concern to malaria epidemiologists, indicating the urgent need to identify and develop alternate compounds capable of curing resistant malaria. If there was more information on relationship between in vitro antimalarial sensitivity and in vivo response to treatment, dose regimens of quinine could be improved. Therapeutic concentrations of quinine need to be defined in different countries where sensitivity of *P. falciparum* to the cinchona alkaloids varies. Quinine should not be used alone. One of the most inexpensive ways of searching new lead antimalarial compounds for drug development would be to screen the drugs which have been developed for other diseases and evaluate their antimalarial potentials, either alone or in combination with other drugs. Searching of novel drugs empirically is expensive in time and resources. Rational drug design is feasible if modes of action of drugs is known, so its highly desirable to demonstrate how the proven drugs function at molecular level. Discovery of the molecular mechanisms causing resistance is of utmost importance in order to impede, reverse or contain the advent of resistance. Search for antirelapse and transmission blocking drugs should be a priority. New drug combinations should be explored to which emergence of resistance is extremely difficult, this strategy might bear fruits quickly, specially search for synergistic combinations. Efforts should continue for the search of less toxic 'resistance reverser' combinations. Ability of some oligos to revert DHFR resistant mutants to sensitive lines in vitro by site directed mutagenesis should be tried as a first step.
Drugs toxic to plasmodia and capable of evoking immunogenic responses against parasite will be of special interest. Potent new antimalarial combinations to be developed should be inexpensive, nontoxic and administered under strict prescription to limit their misuse. There should be a separate class of drugs for prophylaxis. Proper drug deployment, marketing and monitoring strategies will have to be worked out.
Table 1.1 Positions of amino acid residues that are different in pyrimethamine and cycloguanil sensitive or resistant isolates in the DHFR segment of the *P. falciparum* genome*.

<table>
<thead>
<tr>
<th>Susceptibility of the isolate</th>
<th>Amino acid residue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyrimethamine</td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>Ala.</td>
</tr>
<tr>
<td>R</td>
<td>Ala.</td>
</tr>
<tr>
<td>S</td>
<td>Val.</td>
</tr>
<tr>
<td>HR</td>
<td>Ala.</td>
</tr>
<tr>
<td>R</td>
<td>Ala.</td>
</tr>
<tr>
<td>HR</td>
<td>Ala.</td>
</tr>
</tbody>
</table>

| Cycloguanil                  |                    |
| S                            | Asn.               |
| S                            | Asn.               |
| R                            | Asn.               |
| S                            | Cys.               |
| R                            | Cys.               |
| HR                           | Cys.               |
| R                            | Cys.               |
| HR                           | Cys.               |

16  51  59  108  164

S = sensitive, R = resistant, HR = highly resistant

* compiled from the literature
Figure 1.2 Composition of 'quinine' from past to present
Chapter 2

SUSCEPTIBILITY OF PLASMODIUM FALCIPARUM ISOLATE AND A CLONE TO A FEW ANTIMALARIAL DRUGS IN VITRO

Abstract: In vitro susceptibility of a local Plasmodium falciparum isolate together with one of its fastest multiplying clone to some known natural antimalarial drugs (quinine and artemisinin) and commonly used synthetic drugs (chloroquine, amodiaquine, pyrimethamine and cycloguanil) has been performed, using the modified 48 hour test method. Erythrocytic stages of the parasites have been found to be resistant to chloroquine and sensitive to all other natural and synthetic drugs evaluated. Advantages of in vitro bioassay methods for drug susceptibility testing has been discussed. Growth rates of the parasites have been found to be comparable to those of the parasites maintained in cultures in non-endemic areas, implying that serum supplement (obtained locally) employed in the medium for cultivation has no adverse effect on growth and multiplication of the parasites.

INTRODUCTION

The efficacy of drugs against falciparum infections has traditionally been evaluated by observing the clearance of parasites from the blood stream following administration of a drug in a subject. If the parasitemia is cleared with no subsequent recrudescence the drug is considered effective and the infecting strain regarded sensitive to the administered drug. It is obvious from this type of experimental data that some strains of parasites are more sensitive than others to the drug. Marked differences in susceptibility are sometimes observed, particularly in strains of diverse geographical locations. However, with the emergence of chloroquine-resistant strains of falciparum in 1960s, need for defining drug resistance in malaria and standardizing procedures for assessing the varied responses of P. falciparum to chloroquine and other antimalarials was strongly felt. Drug resistance in malaria is defined by WHO (1967) as the "ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within the limits of tolerance of the subject". Although this definition can logically be extended to include all plasmodial stages, it has generally been restricted to describe the drug susceptibility of asexual forms, presumably because this stage in the life cycle of the parasite produces acute clinical symptoms observed during
the course of malaria infection. Evaluation of drug resistance procedures in vivo was proposed by WHO first in 1967 and then slightly modified in 1973 (WHO, 1967, 1973). The obvious problems associated with in vivo determination of the drug susceptibility of *P. falciparum* has led to the development of simple in vitro tests which can be easily performed both in laboratory and field conditions (Rieckmann and Lopez Antunano 1971). The original in vitro microtest method has been the most successfully applied diagnostic test for chloroquine-resistance and was designated by the WHO as a standard test in 1979 (WHO 1979). Procedure of Trager and Jensen (1976) for in vitro cultivation of falciparum stages has given further impetus to modify and develop methods for in vitro determination of susceptibility not only to chloroquine but also to other antimalarial drugs (Nguyen-Dinh and Payne 1980). The results of in vitro assessment in most cases show excellent concordance with the results of their assessment with in vivo system. In vitro method has superseded the in vivo procedure for assessment of drug susceptibility in some situations. Evaluation of the drug susceptibility of *P. falciparum* is now not only confined to those locations where drug resistance is a problem but test are also carried out in all endemic areas where falciparum infections exist. Such susceptibility profile of local strains to antimalarials provide a baseline data against which subsequent changes in the susceptibility of parasites can be measured. Susceptibility status of local strains of *P. falciparum* to new drugs should also be determined in areas where these drugs might be used in future. Malaria is acknowledged as an exclusively local phenomenon and any control programme has to be meticulously designed taking into consideration the susceptibility profile and dynamics of resistance (if known) of the local falciparum fauna to the antimalarial drugs. Over the last few years we have established in vitro cultures of several *P. falciparum* isolates originating from Delhi. Clones have been derived from some of these isolates. Susceptibility profile of some isolates and clones has been determined in vitro to commonly used antimalarial drugs. In
this section sensitivity of an isolate and a clone, which have been employed in all
the subsequent work of the present study, to some antimalarials has been described.
In vitro cultivation procedures and their advantages in drug susceptibility testing
have been discussed.

MATERIALS AND METHODS

Biological materials

Parasites: The isolate FCD-4 employed in the present study was procured locally
from a malaria infected patient reporting to a malaria clinic of malaria research
centre (MRC), Delhi. About 2 ml of blood was drawn aseptically, with the consent
of patient infected with *P. falciparum*, by venous puncture into a sterile heparin-
ized vial. The blood sample was transported to the laboratory for in vitro cultiva-
tion of erythrocytic stages within an hour. Soon after, the cultures were initiated
in vitro using candle-jar method of Trager and Jensen (1976). Once the isolate got
established in culture, clones were derived from it using limiting dilution method
(Rosario 1981). Several clones were realized and a few characterized with regard
to their multiplication ability in vitro. A clone F-56, one of the fastest growing
clone obtained was chosen for further studies.

Red blood cells and serum: Human erythrocytes and serum used for cultivation
of parasites were purchased from commercial blood banks. All blood products
used, were certified to be free from malaria, hepatitis, HIV etc. A unit of blood
(about 350 ml) obtained in acid citrate dextrose or citrate phosphate dextrose was
dispensed aseptically in 50 ml aliquots and stored at 4 °C. The stored blood was
used upto four weeks. Erythrocytes of A+ blood group were used in all studies.
For serum, 2-3 units of AB+ blood at a time were collected without any anticoagu-
lant. It was stored at 4 °C atleast overnight to retrieve maximum amount of
serum. The serum was harvested by centrifugation of this stored coagulated blood
and distributed in 40 ml aliquots in sterile flasks. These serum samples were
kept at -20 °C till further use. Prior to use the stored serum was thawed and heat inactivated by keeping it for 30 min at 56 °C.

**Reagents**

*Medium:* For one litre of medium 10.4 g of powdered RPMI-1640 with L-glutamine but without bicarbonate (obtained from Sigma) was dissolved in 900 ml of glass redistilled water. To this 5.94 g of HEPES buffer and 40 mg of gentamicin were added (RP-C). The above components were thoroughly mixed with the help of a magnetic stirrer and the final volume adjusted to 960 ml. This medium was immediately filter sterilized through 0.45μ presterilized millipore filter under negative pressure, aseptically in a flow hood. The sterilized medium was dispensed in 100 ml volumes into sterile screw cap bottles for storage in refrigerator. Each bottle was numbered and dated. The pH of the buffered medium was made to 7.2 just before use by adding sterilized 5% sodium bicarbonate solution at the rate of 4.2 ml per 96 ml (RP+C) medium. The RP+C medium was supplemented with 10% (v/v) heat inactivated serum to be used as complete medium (RPS) for cultivation. Complete medium could be stored at 4 °C upto one week in closed bottles. The RP-C was consumed within four to five weeks.

*Cryoprotectant:* It was prepared by adding 70 ml glycerol to 180 ml of 4.2% sorbitol in 0.9% NaCl and filter sterilized to be used as cryoprotectant solution for freeze-storing of parasites.

*Thioglycollate medium:* Thioglycollate medium, 29.8 g (powder) was mixed in 1 litre of glass distilled water and heated slowly so as to dissolve it completely. This solution was dispensed in 10 ml volumes into glass tubes with cotton plugs and autoclaved at 15 lbs for 20 min. Tubes were stored at 4 °C for subsequent testing of bacterial contamination in medium or blood products.

**Drug solutions** The stock solutions concentration and methods of preparation of the various drugs employed in this study are tabulated below.
### Drug Source Concentration of Stock Solvent

<table>
<thead>
<tr>
<th>Drug</th>
<th>Source</th>
<th>Concentration of Stock</th>
<th>Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroquine</td>
<td>Sigma Chemicals</td>
<td>40 mg/ml base</td>
<td>Water</td>
</tr>
<tr>
<td>Amodiaquine</td>
<td>Sigma Chemicals</td>
<td>$10^{-2}$ M</td>
<td>Water</td>
</tr>
<tr>
<td>Pyrimethamine</td>
<td>Sigma Chemicals</td>
<td>$10^{-2}$ M</td>
<td>Lactic acid</td>
</tr>
<tr>
<td>Cycloguanil</td>
<td>ICI Pharmaceuticals*</td>
<td>$10^{-2}$ M</td>
<td>Ethanol</td>
</tr>
<tr>
<td>Quinine</td>
<td>WHO*</td>
<td>10 mg/ml</td>
<td>Sulfuric acid</td>
</tr>
<tr>
<td>Artemisinin</td>
<td>WHO*</td>
<td>1 mg/ml</td>
<td>DMSO</td>
</tr>
</tbody>
</table>

* gifts from these agencies

Each of these stock drug solutions was filter sterilized through 0.2 mm cellulose acetate millipore sterile disposable syringe filter and stored at -20 °C. Test or working solutions were prepared by appropriately diluting the stock solutions with complete medium (RPS). All the test solutions were kept at 4 °C.

### PROCEDURES

#### Maintenance of *P. falciparum* parasites in vitro

**Sterility test:** All media, blood and glassware must be sterile for culture work. The glasswares were autoclaved and baked. The sterility of the glassware was ensured by the pressure and heat indicator tapes. To test the sterility of media and blood products, 1ml of test solution was mixed with the sterile thioglycollate medium. The tube was incubated at 37 °C for 4 days. If thioglycollate solution remained clear, the tested product was considered free of bacterial contamination.

**Red cell preparation** (washed erythrocytes): Human erythrocytes obtained from blood bank were centrifuged at 2500 rpm for 5 min. Supernatant and buffy coat were aspirated out. The pellet was resuspended in 5-10 volume of RP+C medium and spun again to remove any residual anticoagulant. The cells were washed twice this way and finally the pellet of red cells was resuspended in an equal volume of complete medium (RPS) to give 50% cell suspension.

**Cultivation of parasites in vitro:** Parasites were cultivated using the Petridish candle jar method of Trager and Jensen (1976). The parasite material was drawn into a sterile syringe from a flow vessel, in which stock cultures of parasites were
routinely maintained in the laboratory (Trager 1979). The material was transferred to a graduated centrifuge tube and centrifuged at 2000 rpm for 10 min. Supernatant was discarded and packed cells were resuspended in equal volume of complete medium. Thin blood smear was made from this 50% infected cell suspension. The slide was fixed in methanol and stained in 5% Giemsa's stain prepared in 0.067 M phosphate buffer. The slides were air dried and microscopically examined under oil immersion magnification (1000 X). Differential parasite count was made to determine the extent of dilution required, if any. Parasite material was diluted with 50% suspension of non-infected erythrocytes. Cultures were initiated with less than 1% of infected erythrocytes. Appropriately diluted parasite material of 50% cell suspension, 2 ml of which was mixed with 10 ml of complete medium (RPS) so as to give a final suspension or hematocrit of 8%. This material (about 12 ml) was dispensed in a glass Petridish (10 cm in diameter). The Petridish was placed in a desiccator equipped with a stopcock. The gas phase was generated by burning a white candle in the desiccator jar holding the Petridishes until the flame extinguished; the stopcock was closed immediately. The candle jar, holding parasite material in Petridishes, was placed at 37 °C in an incubator. The medium was removed daily by gently tipping the Petridish and aspirating off the medium. All this was done under sterile conditions in a flowhood. The dishes were then replaced in the candle jar. Culture growth was routinely monitored every 48 hour from thin blood smears stained in Giemsa solution. Differential parasite count was made by counting at least 5000 erythrocytes. If parasitemia was found to be more than 5% subcultures were prepared.

Cryopreservation: Cultured parasite material was periodically stored in liquid nitrogen for recultivation. Parasites were cryopreserved by a modified method of Rowe et al. (1968). Cryopreservation of parasite material was done when number of parasites per 100 red cells exceeded 5 in a Petridish and majority of para-
sites were in ring stage. The parasite material was transferred to a graduated centrifuge tube and centrifuged at 1500 rpm for 10 min. Supernatant was discarded and the packed cells mixed gently with an equal volume of cryoprotectant and 1ml each of this suspension was transferred into sterile, screw-capped plastic cryovials. These vials were immediately dipped in liquid nitrogen and stored in liquid nitrogen cryocan.

**Resuscitation of parasites:** Cryopreserved parasites were revived by bringing the cryovial from liquid nitrogen to a 37 °C water bath (momentarily loosening the cap to vent any compressed nitrogen). On thawing, contents of the vial were transferred to a centrifuge tube, spun at 1500 rpm for 5 min, supernatant discarded and packed cells suspended in an equal volume of 3.5% sterile sodium chloride solution. The saline suspension of cells was recentrifuged to discard the saline, packed cells washed twice with complete medium and finally made to 50% cell suspension with complete medium. To this, equal volume of freshly washed uninfected 50% suspension of erythrocytes was mixed. The mixture was made to 4% hematocrit by adding complete medium supplemented with 15% heat inactivated pooled serum in a glass Petridish (5 cm diameter) and parasites grown by candle jar method.

**Susceptibility test method**

Erythrocytic schizontocidal activity of each compound at graded concentrations was evaluated in vitro using the modified 48 hr test method of Nguyen-Dinh and Payne (1980). The parasite material was drawn from stock continuous culture, centrifuged and pelleted cells suspended in 2.5 volume of 5% sorbitol and incubated at room temperature for 7 min. Mostly the ring stage parasites survive this treatment. The sorbitol treated parasites were washed thrice with RP+C medium. The pellet was finally resuspended in an equal volume of complete medium to give 50% suspension of infected erythrocytes. This infected material was appropriately diluted with 50% washed, uninfected erythrocytes so as to get
the final parasitemia of less than 1% (zero hour) to be used as seeding material for susceptibility tests. Aliquots of 20 μl of this seed material was added to each of the series of wells, in 24 well sterile plastic tissue culture plates, holding 480 μl of complete medium with or without drug. The final erythrocyte suspension in these experimental wells was thus 2%. The experiment was done in duplicate for each drug concentration. After thoroughly shaking the multiwell plates to ensure resuspended and uniform settling of erythrocytes, the plates were placed in candle jar and incubated at 37 °C. After 24 hours of incubation, medium in each well was replaced with or without drug. Drug was included at same concentration as initially and plates returned to candle jar. Cultures were under continuous drug pressure for 48 hour, at the end of this period a thin blood film from each of the well holding control and drug treated parasite material was made. Slides were stained with 5% Giemsa solution and number of parasites counted by enumerating atleast 5000 erythrocytes. Percentage inhibition of parasites in relation to control was calculated. MIC, IC_{50} and IC_{90} values were determined from semilog plot of percentage inhibition against drug concentration.

RESULTS

In vitro cultivation of *P. falciparum*

A vial each of cryopreserved material of *P. falciparum* isolate FCD-4 and clone F-56 was retrieved from the liquid nitrogen storage container to initiate in vitro cultures of these parasites separately. Once the parasites started multiplying to an extent of atleast four fold in an erythrocytic cycle (from starting parasitemia of less than 1%) they were further maintained in a routine medium (RP+C) supplemented with 10% serum (RPS). This usually took not more than 6 days of culture, if properly cryopreserved material was used for resuscitation. Freeze preserved material when initiated into culture retained synchronous multiplication for first one or two schizogonic cycles and subsequently grew asynchronously. An
erythrocytic cycle is of about 48 hours duration. During this period intraerythrocytic parasites undergo morphological changes leading to the formation of ring, trophozoite and schizont stages. Rupture of a schizont releases merozoites, the only extracellular form, which reinitiates the erythrocytic cycle on invading an uninfected red cell. A representative Giemsa stained thin blood film preparation from cultured parasite material showing various asexual and sexual stages is presented in Figure 2.1. The ring stage is the youngest intraerythrocytic stage of the parasite occupying approximately one fifth of the red cell area. Host red cells at this stage were least modified with regard to morphology or membrane permeability. A typical 'signet' ring stage occupied first 18-24 hours of an erythrocytic cycle before being transformed into the trophozoite stage. A growing trophozoite could be seen as a small round mass of blue cytoplasm with one red chromatin dot or area and a small clump of brown pigment. The more mature or nucleus dividing stage is called schizont which looks like a large trophozoite containing more than one chromatin dot. Each nucleus with cytoplasm surrounding it is called merozoite, may be seen in a schizont. Fully formed merozoites released from a mature schizont can be seen in the Figure 2.2. Occasionally developing sexual stages, called gametocytes and often multiple infection of red cell can also be found in cultures. A typical distribution of different erythrocytic stages in a culture started from ring stages after first and second schizogonic cycle is depicted in Table 2.1.

Table 2.1. A differential parasite count made at the end of first and second schizogonic cycles of in vitro cultivated parasites in multiwell plates with starting hematocrit of 4%, showing typical profile of erythrocytic stages in the cultures.

<table>
<thead>
<tr>
<th>Schizogonic cycle</th>
<th>FCD-4</th>
<th>F-56</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R*</td>
<td>T</td>
</tr>
<tr>
<td>0 hour</td>
<td>36</td>
<td>0</td>
</tr>
<tr>
<td>First</td>
<td>110</td>
<td>18</td>
</tr>
<tr>
<td>Second</td>
<td>259</td>
<td>48</td>
</tr>
</tbody>
</table>

* R= rings, T= trophozoites, S= schizonts, TP= total parasites, %P= % parasitemia
Usually a parasite produces by asexual multiplication (schizogony) 8 to 24 merozoites. But only some of these merozoites succeed in invading and multiplying further. This determines the rate of multiplication and to monitor the growth of parasites, thin smears were made after every 48 hours for two consecutive cycles from each of the well holding either FCD-4 or F-56 culture material. Parasitemia was enumerated from the stained slides by counting about 5000 erythrocytes. Multiplication factor (MF) and parasite multiplication rate (PMR) were determined using the following formula (Brokelman et al. 1985):

For the first schizogonic cycle \( MF = \frac{N \text{ (day 2)}}{N \text{ (day 0)}} \)

where \( N \) is the number of parasites per 5000 erythrocytes on the day indicated.

Thus the MF from day 2 to day 4 was \( = \frac{N \text{ (day 4)}}{N \text{ (day 2)}} \)

Because multiplication is exponential (theoretically for first few cycles), a logarithmic transformation was used as follows:

\[ \log MF = \log N \text{ (day 4)} - \log N \text{ (day 2)} \]

Log MF has been referred as the parasite multiplication rate (PMR). Results regarding MF and PMR for the isolate and clone are presented in Table 2.2.

**Table 2.2** In vitro parasite multiplication as determined by multiplication factor (MF) and parasite multiplication rate (PMR) in multiwell tissue culture plates. (refer to the text for explanation of MF and PMR)

<table>
<thead>
<tr>
<th>Parasite</th>
<th>Erythrocytic schizogonic cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>First</td>
</tr>
<tr>
<td></td>
<td>MF</td>
</tr>
<tr>
<td>FCD-4</td>
<td>4.36</td>
</tr>
<tr>
<td>F-56</td>
<td>5.96</td>
</tr>
</tbody>
</table>
Parasites were periodically cryopreserved for subsequent use from culture plates having more than 7-8% parasitemia with majority of parasites in the ring stage. This type of freeze-stored material revived fast, mostly within 4 to 6 days of resuscitation cultures could be grown in a routine medium supplemented with 10% serum.

**In vitro susceptibility to antimalarials**

The response of the parent isolate FCD-4 and one of its derived clone F-56 to the various antimalarials evaluated in vitro were as follows:

*Chloroquine:* Figure 2.3 shows the drug dose response for chloroquine in the isolate FCD-4 and clone F-56 after 48 hours of incubation. The IC_{50} and IC_{90} values of chloroquine were found to be 0.018 μg/ml and 0.07 μg/ml for FCD-4 and 0.015 μg/ml and 0.027 μg/ml for clone F-56.

*Amodiaquine:* Susceptibility of the parasites to various concentrations of amodiaquine is illustrated in Figure 2.4. Interpolation of the drug dose response curve to determine concentrations that caused 50% and 90% inhibition of parasite growth gave values of 6.8 x 10^{-9} M and 5.4 x 10^{-8} M for FCD-4 and 1.7 x 10^{-9} M and 7.4 x 10^{-9} M for clone F-56. The clone was found to be slightly more sensitive to this drug since complete inhibition of clonal parasites was achieved at a concentration of 10^{-8} M whereas in the isolate it was observed at 10^{-7} M of drug concentration.

*Pyrimethamine:* Sensitivity of the isolate FCD-4 and clone F-56 to graded concentrations of pyrimethamine in vitro is presented in Figure 2.5. Drug concentration of 10^{-8} M or more uniformly affected the morphological appearance of schizonts, as seen from 96 hours Giemsa stained blood films in both the parent isolate FCD-4 and clone F-56. Schizonts showed fragmented chromatin dots of unequal size scattered over a large area. Normal merozoite formation was prevented resulting in the inhibition of parasite multiplication. In control wells 7 to 8 fold increase in parasitemia was observed by the end of 96 hours. The IC_{50} and IC_{90}
values of the drug derived from the drug dose response curve were $1.5 \times 10^{-9}$ M and $4.6 \times 10^{-9}$ M for FCD-4 and $1.5 \times 10^{-9}$ M and $3.6 \times 10^{-9}$ M for F-56. Apparently lactic acid used for drug preparation at concentrations present in the test solutions had no effect on parasite multiplication. This confirms the finding of Richards and Maples (1979).

**Cycloguanil:** Parasite counts made at the end of 96 hours of the experiment revealed that multiplication of the parent isolate FCD-4 and clone F-56 was completely inhibited in vitro at $5 \times 10^{-9}$ M and $5 \times 10^{-10}$ M concentration of cycloguanil. Percent inhibition of parasite growth in relation to control was plotted against log concentration of drug to determine IC$_{50}$ and IC$_{90}$ values. The isolate FCD-4 and clone F-56 showed marked sensitivity to cycloguanil. The IC$_{50}$ and IC$_{90}$ values derived from the drug dose response curve (Figure 2.6) were $8.4 \times 10^{-11}$ M and $6.8 \times 10^{-10}$ M for FCD-4 and $7.2 \times 10^{-11}$ M and $2.7 \times 10^{-10}$ M for the clone F-56. Inhibitory effect of the drug on parasite growth was observed even at very low concentrations. The morphological appearance of cycloguanil sensitive parasites was very similar to that observed in the case of pyrimethamine. These abnormal parasites appeared enlarged and contained fragmented red staining nuclear material. Good growth of parasite was observed in control wells at the end of 96 hours.

**Quinine:** The effect of quinine on erythrocytic stages of the clone F-56 and isolate FCD-4 in vitro is illustrated in Figure 2.7. The IC$_{50}$, IC$_{90}$ and MIC values of the drug for the parasites derived from the drug response curve are 0.02 µg/ml, 0.052 µg/ml and 0.06 µg/ml for clone F-56 and 0.01 µg/ml, 0.047 µg/ml and 0.06 µg/ml for the isolate FCD-4.

**Artemisinin:** The response of clone F-56 to graded concentrations of artemisinin is presented in Table 2.3. Complete inhibition of erythrocytic multiplication was obtained at a concentration of 10 ng/ml of the drug. The concentrations of the drug that caused 50% and 90% inhibition of parasite growth with respect to control derived from the tabulated data (Table 2.3) are 0.0019 µg/ml and 0.0058 µg/ml.
respectively.

Table 2.3  In vitro susceptibility of a P. falciparum clone, F-56 to artemisinin. The parasites were under drug pressure for 48 hours. Starting parasitemia was less than 1% and hematocrit 4%. Slides were enumerated for parasitemia at the end of 48 hours.

<table>
<thead>
<tr>
<th>Drug concentration (µg/ml)</th>
<th>% parasitemia</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>2.66</td>
<td>0</td>
</tr>
<tr>
<td>0.01</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>0.005</td>
<td>0.37</td>
<td>86.09</td>
</tr>
<tr>
<td>0.001</td>
<td>2.07</td>
<td>22.18</td>
</tr>
<tr>
<td>0.0005</td>
<td>2.37</td>
<td>10.9</td>
</tr>
<tr>
<td>0.0001</td>
<td>2.71</td>
<td>0</td>
</tr>
</tbody>
</table>

DISCUSSION

One of the most widely used applications based on continuous cultivation of erythrocytic stages of falciparum malaria (Trager and Jensen 1976) has been the in vitro determination of susceptibility of parasites to various antimalarial drugs around the world, more so in endemic locations. The popularity of these methods is obviously because of several advantages they offer over in vivo procedures. The 'standard' WHO Field Test (1973) for instance which is used to determine the susceptibility of P. falciparum infection in the subject requires 3 days of drug administration and 7 days of follow up period to ascertain the sensitivity of infecting parasites. Low levels of resistance can not be detected by this in vivo procedure.
Modified 'extended' Field Test requiring 28 days of follow up is employed to determine the gradation from sensitive (S) to R I, R II or R III (high) level of resistance to chloroquine. Prolong follow up period with precautions for preventing any re-infection during this duration are limiting factors to carry out this test, particularly in endemic locations. The in vitro methods of determining the sensitivity of an isolate require much less time and can be easily completed in 2 to 3 days using very little blood from the infected person. The response of falciparum infection to drug treatment may differ from one individual to another depending upon the immune status of the individual (Powell et al. 1964; Waliker and Lopez Antunano 1968). The hosts immune and other factors do not affect the outcome of results in vitro evaluation, thus permitting a more accurate evaluation of the intrinsic inhibitory properties of the drug on the parasites. However, serum supplement used, specially those procured from endemic regions, might interfere with correct assessment of the inhibitory properties of the drug, as serum from various endemic regions are known to contain antibodies capable of retarding the growth of cultures in vitro (Chulay et al. 1981; Jensen et al. 1982). To overcome this possibility each serum unit should be screened for the presence of malaria specific antibodies or the serum used should be heat inactivated to nullify the influence of these serum factors on the cultures. All through the present study the latter option was employed. This minimized the interference of serum factors in drug response studies of the parasite. Another drawback with in vivo evaluation is concerning uncertainty of complete absorption and retention of the drug in the subject. Assessment of complete uptake and presence of the correct amount of the drug in the blood of infected person requires use of time consuming, elaborate methods like HPLC. Applications of in vitro techniques have thus greatly improved the overall efficiency and precision of drug sensitivity tests. It is now possible to process and evaluate large number of compounds on different strains of falciparum simultaneously, thus providing broader range of information with respect to susceptibil-
ity to various drugs. This has been made possible also because these techniques are relatively inexpensive compared to experimental animal model systems which require more space, equipment and manpower. In vitro cultures have made it possible to study clonal populations of parasites and this will contribute towards better understanding of genetic analysis of drug action and considerably facilitate molecular/biochemical studies on the mechanism of drug action. In vitro systems permit study of structural-activity relationship of compounds with great precision and relative ease, thus allowing the selection of lead compounds with greatest intrinsic antimalarial activity very rapidly. Evaluation of potential drug interaction for synergism, antagonism or indifference has been made simpler by in vitro methods, enabling the selection of favourable combinations. By employing multi-drug resistant strains in culture it is now possible to look for compounds which are not cross-resistant with existing antimalarial drugs. Finally it is possible in vitro studies to evaluate parent compounds and known or putative metabolites of the parent compounds separately, especially since only small quantities of each are needed. There are also important limitations associated with the use of in vitro methods. The most frequently cited limitation is the inability to detect the potential antimalarial activity of those compounds which require in vivo metabolism to an active state, for example the antimalarial drug proguanil which is inactive in vitro system. Similarly certain compounds which require some form of host interaction such as enhancement of host immunity may prove to be inactive when assessed in vitro. Another limiting characteristic of in vitro method is the artificial time course chosen for drug exposure, which is usually at a constant level of concentration for a relatively short period of time. Certain drugs which have been identified as effective antimalarials may ultimately prove to be toxic to the host or might have very little safety margin. Many compounds will also prove to be insoluble in the media used for culturing the parasites in vitro. Others will be subject to artifacts peculiar to in vitro systems such as adherence to the glass or plas-
tic dishes in which the experiments are carried out. The above considerations lead to the conclusion that though the availability of in vitro culture techniques is important for antimalarial drug research, they are neither a substitute nor a replacement for in vivo or basic biochemical methods. A well-designed drug development programme must carefully integrate these varied approaches, recognising their respective advantages, limitations and their mutual potential contributions towards the goal of identifying and characterising useful new antimalarial drugs.

Parasite multiplication factor in our study was comparable with the isolates and clones being cultivated elsewhere even in non-endemic areas. There were no crisis forms observed in cultures which have been reported from some holoendemic regions. The presence of malaria specific antibodies in the blood have been implicated for the presence of crisis form (Reese et al. 1981; Jensen et al. 1982, 1984). There had been no excessive conversion rate of asexual forms to sexual stages in the culture as has been the case in most endemic regions employing hyperimmune serum (Jensen et al. 1984). All these observations peculiar to parasite growth and multiplication in the malaria endemic regions are contrary to the observations made in our cultures which confirm that the blood component used by us for cultivation were free from most of the factors involved in generating these characteristics. Oduola and coworkers (1992) have also found that plasma from semi-immune persons is suitable for continuous cultivation and drug susceptibility testing of *P. falciparum*.

The first simple in vitro test for assessing the drug susceptibility of *P. falciparum* parasites drawn from venous blood of an infected individual was developed by Rieckmann et al (1968). The test relied on a basic observation that parasites could survive in vitro system for short duration of at least 30 hours. This fact was exploited in measuring the extent to which maturation of ring forms to schizonts was inhibited after incubation of parasitized blood in various drug concentrations for a period of 24 hours. This short term culture system provided a quick and
reliable diagnostic tool to differentiate chloroquine resistant and sensitive isolates both in laboratory and field conditions (Rieckmann and Lopez Antunano 1971; Valera and Shute 1975; Palmer et al. 1976; Ebisawa et al. 1976; Sucharit et al. 1977). Usefulness of this method culminated in the development of a test kit for monitoring the sensitivity of \textit{P. falciparum} isolates to chloroquine by the WHO (1979). Using this kit one could even assess the level of resistance ranging from sensitive to highly resistant R III forms of the parasites. This test has also been used for other antimalarials. The main shortcoming of this macro-method is that it requires about 10 ml of venous blood which is not readily given by the patient, more so if the subjects are children. Development of microtechnique using capillary blood specimen (Rieckmann et al. 1978), which can be obtained by a finger prick, has made the susceptibility test easier and can be performed by drawing little blood from even young children. Period of incubation in the micromethod can be extended to 48 hours without change of medium, so that one can even observe reinvasion of the merozoites (Rieckmann 1980; Yisunsri and Rieckmann 1980). This technique has replaced macro test specially in the field with the development of inexpensive battery operated out door incubators (Eastham and Rieckmann 1981). Considering the benefits of incubating parasites under drug pressure for longer duration in determining the susceptibility of falciparum isolates, Nguyen-Dinh and Trager (1980) described a new 48 hour test system. Infected red blood cells, either from cultures or from clinical specimens (Nguyen-Dinh et al. 1981) were diluted in a 50% suspension of fresh red blood cells in culture medium to obtain a parasite density of 0.1%-0.8%. The resulting parasitized erythrocyte suspension was then further diluted in culture medium, with or without drug added, to a 2% erythrocyte suspension. A similar test method of 72 hours has been described by Kramer and Siddiqui (1981) with 5% erythrocyte suspension. In the present study a modified 48 hour test method of Nguyen-Dinh and Payne (1980) has been employed in determining the susceptibility of para-
sites to antimalarial drugs in vitro, because this test method is reliable, reproducible and results are not modified by slight variations in starting parasitemia, erythrocyte suspension, blood group of the red cells used, age of the erythrocytes, inoculum size etc.

According to Nguyen-Dinh and Trager (1980) for a strain sensitive to chloroquine complete inhibition of growth occurs at 0.01 μg/ml and for a resistant strain at 0.1 μg/ml. From IC_{50} and IC_{90} values derived from the drug response curve it can be inferred that both the parent isolate and the clone evaluated are resistant to chloroquine, the isolate being more resistant since at a concentration of 0.06 μg/ml complete inhibition is observed in the clonal parasites whereas only 86% inhibition was observed in the parent isolate. In control wells in which parasites were incubated in drug free medium good growth was obtained with 4 to 5 fold increase in parasitemia in 48 hours, which is comparable with the growth observed by Nguyen-Dinh and Trager (1980). Amodiaquine and chloroquine are both derivatives of 4-amino-7-chloroquinoline and differ only in the nature of the group attached to the 4 amino position, amodiaquine has a substituted benzene ring at the position, while chloroquine has a branched chain aliphatic substituent. The parasites evaluated have been found to be sensitive to amodiaquine while extremely resistant to chloroquine. Similar observations have been made by other workers (Thaithong et al. 1983; Watkins et al 1984; Looareesuwan et al. 1985) in strains of *P. falciparum* from different geographical regions, and also it has been observed that isolates found to be resistant to chloroquine develop resistance to amodiaquine sooner or later (Draper et al. 1988). Response of parasites to both the DHFR inhibitors evaluated was found to be similar. This is perhaps expected since both pyrimethamine and cycloguanil are known to act by affinity binding to the dihydrofolate reductase of the parasite which occupies a position in the cellular biosynthesis of the malaria parasites (Ferone et al. 1969). Smalley and Brown (1982) estimated that parasites obtained from clinical samples growing in 10^{-7} M
pyrimethamine or more are likely to be resistant at least to some degree in 24 hour test. Concentration of $1 \times 10^{-8} \text{M}$ of pyrimethamine in a 48 hour test in vitro by Richards and Maples (1979) has been reported to be both inhibitory and cidal. As the morphological criterion has been used to determine the effect of the compound on *P. falciparum*, sometimes it becomes difficult to differentiate between the cidal and inhibitory effect on the parasites in a stained slide. In order to eliminate this ambiguity, the parasites were further cultivated for next 48 hours in drug-free medium and percentage inhibition of parasites calculated at 96 hours. The IC$_{50}$ and IC$_{90}$ values determined both at the end of 48 and 96 hour experiments clearly indicate that isolate (FCD-4) and clone (F-56) tested are highly sensitive to pyrimethamine and cycloguanil. Information on the relationship between in vitro schizontocidal activity and in vivo response to treatment are lacking for quinine and artemisinin. Quinine is being used in India for emergency medicament of malaria but so far no reports have emanated regarding treatment failure with this drug, indicating the sensitivity of the parasites to quinine. Mean plasma level of 1.5 μg/ml of quinine base is reached in a person 4 hours after ingesting a single dose of 300 mg of quinine (Saggers et al. 1970). Complete inhibition of parasites were obtained at much lower concentrations, clearly indicating that parasites used were sensitive to quinine. This experiment has also generated a baseline data of these parasites for quinine susceptibility. Artemisinin has not been used for malaria treatment in India, therefore parasites are expected to be sensitive to this drug. The mean plasma level of 110 ng/ml attained at about 7 hours in humans taking the curative dose of 10 mg/kg body weight (Zhao and Zhang 1985, 1986) of artemisinin is much higher the concentration of the drug needed to prevent the multiplication of the parasites used in vitro, indicating that the parasites of the clone F-56 are sensitive to artemisinin and we have baseline values of susceptibility of this parasite line to a drug which may be used in future for malaria treatment.
Figure 2.1 A typical Giemsa stained thin blood film made from asynchronous culture showing various asexual erythrocytic stages (R= ring, T= trophozoite, S= schizont) and a sexual stage (G= gametocyte). (X 1200)

Figure 2.2 A Giemsa stained thin blood film depicting free merozoites released from a ruptured schizont.
Figure 2.3  Drug dose response curve for chloroquine in the *P. falciparum* isolate FCD-4 and cl F-56 after 48 hours of incubation in vitro (performed in 24-well sterile plastic tissue culture plat
Figure 2.4  Response of the *P. falciparum* isolate FCD-4 and clone F-56 to various concentrations of amodiaquine in vitro.
Figure 2.5  Pyrimethamine sensitivity of the *P. falciparum* isolate FCD-4 and clone F-56 determined in vitro by the modified 48 hour test method.
Figure 2.6  Cycloguanil response curves of the *P. falciparum* isolate FCD-4 and clone F-56 in the modified 48 hour test method.
Figure 2.7  Sensitivity of erythrocytic stages of *P. falciparum* (FCD-4 and F-56) to quinine in vitro.
Chapter 3

CHEMOSENSITIVITY OF A CHLOROQUINE-RESISTANT P. FALCIPARUM ISOLATE TO QUININE-TYPE COMPOUNDS IN VITRO

Abstract: In vitro relative chemosensitivity of erythrocytic stages of the chloroquine resistant P. falciparum isolate to quinine-type compounds has been evaluated. 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 IC₅₀ 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.

INTRODUCTION

The property of cinchona bark to cure fevers of diverse origin was well known to the natives of South America, particularly to the Peruvian and the Bolivian (now Ecuador) Indians, much before the recorded historical date of 1630s when the first European was successfully treated (presumably of malaria) by the powdered bark of cinchona tree (Guerra 1977 a, b.). Causative agent of the disease itself was first discovered and identified by Laveran (1880). The power of hot infusion of the powdered bark has been widely exploited in the treatment of malaria since then. Ever increasing demand of this bark, which could easily be mistaken for that of other trees, especially in the dried up conditions led to frauds, speculative and hoarding tendencies among traders in nineteenth century (Gramiccia 1987). This generated interest in chemical analysis of the cinchona bark. The curative property of the bark was assigned to alkaloid components. The bark of cinchona trees contains a mixture of some 10 alkaloids, but most of them are not crystallizable (Bruce-Chwatt et al 1981). Quinine and cinchonine were the first two alkaloids isolated in crystalline form in 1820 by Pelletier and Caventou (Hofheinz and Merkli 1984). Quinine salt proved to be an excellent antimalarial drug. Although correct structure of quinine had already been suggested by 1908, synthesis of this compound was achieved by Woodward and Doering
MATERIALS AND METHODS

Parasites  Locally collected *P. falciparum* isolate FCD-4, being routinely maintained in the laboratory using candle-jar method of Trager and Jensen (1976) was employed in this study. Isolation, establishment and maintenance in vitro of this isolate has been described in an earlier section of this chapter. The characterization of the isolate demonstrated that FCD-4 was resistant to chloroquine.

Drug solutions  Coded preparations of cinchona alkaloids and their derivatives or mixtures were kindly provided by Dr. P.I. Trigg of the World Health Organization. Detail chemical structure of these compounds is given in Figure 3.1. Identity of the eight preparations used in the present investigation is as follows:

<table>
<thead>
<tr>
<th>Name</th>
<th>Abbreviations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Quinine (base)</td>
<td>Q</td>
</tr>
<tr>
<td>2. Quinidine (base)</td>
<td>Qn</td>
</tr>
<tr>
<td>3. Apoquinine (base)</td>
<td>AQ</td>
</tr>
<tr>
<td>4. Hydroquinine (base)</td>
<td>HQ</td>
</tr>
<tr>
<td>5. Hydroquinidine (base)</td>
<td>HQn</td>
</tr>
<tr>
<td>6. Quinine/Hydroquinine</td>
<td>Q/HQ</td>
</tr>
<tr>
<td>60:40 mixture (bases)</td>
<td></td>
</tr>
<tr>
<td>7. Quinidine/Hydroquinidine</td>
<td>Qn/HQn</td>
</tr>
<tr>
<td>60:40 mixture (bases)</td>
<td></td>
</tr>
<tr>
<td>8. Epiquinine/Epiquinidine sulphate</td>
<td>EQ/EQn</td>
</tr>
<tr>
<td>60:40 mixture</td>
<td></td>
</tr>
</tbody>
</table>

Stock solution of each compound was made by dissolving 1000 mg of the substance in 61.6 ml of 0.1N sulphuric acid and diluted with glass distilled water to 100ml. Further dilutions of the stock solutions were made with HEPES buff-
RESULTS

Chemosensitivity of isolate FCD-4 to each compound was initially evaluated by determining the blood schizontocidal activity in duplicate at 1, 3, 6, 10, 60, 100, 300, 600, 1000 ng/ml concentrations of the compound. Minimum inhibitory concentration (MIC) required to completely eliminate parasites with EQ/EQn (mixture), AQ, Q, HQ was found to be 600, 100, 60, 30 ng/ml, respectively whereas other preparations effectively eliminated all parasites at concentration of 10 ng/ml or less. The results obtained are summarized in the Table 3.1.

Table 3.1 Estimated MIC, IC₉₀ and IC₅₀ values of quinine and quinine-type compounds assayed in vitro against a chloroquine resistant isolate FCD-4 of P. falciparum in 24 well sterile plastic tissue culture plates with starting parasitemia of less than 1%, erythrocyte suspension of 2%. The parasites were under drug pressure for 48 hours.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>*IC₅₀</th>
<th>*IC₉₀</th>
<th>*MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quinine</td>
<td>11</td>
<td>48</td>
<td>60</td>
</tr>
<tr>
<td>Quinidine</td>
<td>4</td>
<td>5.8</td>
<td>10</td>
</tr>
<tr>
<td>Apoquinine</td>
<td>13</td>
<td>78</td>
<td>100</td>
</tr>
<tr>
<td>Hydroquinine</td>
<td>5.6</td>
<td>9.4</td>
<td>30</td>
</tr>
<tr>
<td>Hydroquinidine</td>
<td>2.5</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Quinine/Hydroquinine</td>
<td>3.9</td>
<td>8.4</td>
<td>10</td>
</tr>
<tr>
<td>Quinidine/Hydroquinidine</td>
<td>2.2</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>Epiquinine/Epiquinidine sulphate</td>
<td>140</td>
<td>270</td>
<td>600</td>
</tr>
</tbody>
</table>

* Concentrations are in ng/ml.
tive than quinine at $I_{C_50}$ level. Amount of quinidine needed to completely eliminate parasites in vitro is far below the non-toxic peak quinidine levels achievable in plasma of human subjects. Quinidine has greater systemic clearance and a larger total apparent volume of distribution compared with quinine (White 1987). Plasma concentrations during malaria treatment are therefore lower. In the slightly insensitive strains of *P. falciparum* to quinine, although mostly cases of the RI-type of resistance have been recorded (Cook 1986) quinidine has been re-discovered as more potent antimalarial than quinine. So far there has been no report of quinidine resistance either from field or laboratory studies. Information on the relationship between in vitro antimalarial sensitivity and in vivo response to treatment with quinidine is lacking. This information can greatly improve and define the dose regimens of quinidine in different parts of the world depending on the susceptibility of local strains, thus increasing the safety margin of this drug. Apart from natural cinchona alkaloids the synthetic hydroderivatives of quinidine, quinine and their combinations showed extremely good erythrocytic schizontocidal activity. Advantages of hydroquinine over quinine had also been observed by other workers (Giemsa and Werner 1914). The unnatural isomers of dihydroquinine and dihydroquinidine, the enantiomers or mirror image compounds, were shown by Brosi and colleagues (1971) to have same degree of activity as the natural isomers in *P. berghei* in mice. The four major alkaloids all possess significant blood schizontocidal activity but their relative potencies vary with the species of *Plasmodium* (Hofheinz and Merkli 1984). Differences in apparent activity of cinchona alkaloids in vivo are related to both pharmacokinetic factors in the host and pharmacodynamic factors in the parasite. But evaluation of chemosensitivity in vitro system eliminates host factors effectively. Quinine is evidently not the most effective cinchona alkaloids but has reached a unique position because it just happened to be the first to be crystallized and used in 'pure' form. Also because the cultivation of cinchona trees in South America.
Figure 3.1 Chemical structures of quinine and quinine-type compounds
Chapter 4

CURE WITH CISPLATIN OF MURINE MALARIA INFECTION AND INHIBITION IN VITRO OF A CHLOROQUINE RESISTANT P. FALCIPARUM ISOLATE

Abstract: 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. 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 but 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.

INTRODUCTION

Species of Plasmodium are the causative agents of malaria in man and some other vertebrates. P. berghei is responsible for murine malaria in mice and P. falciparum causes 'malignant tertian' human malaria. Appearance and speedy spread of resistance in P. falciparum to common synthetic antimalarial drugs like chloroquine (Peters 1990b; Bruce-Chwatt et al. 1981), pyrimethamine (Peters 1984; 1990b), proguanil (Chaudhuri and Chaudhuri 1949; Field and Ederson 1949; Peters 1990b), used for treatment are showing an increasing rate of curative failures (Peters 1984; Wernsdorfer 1991). The current grim situation is thus a cause of concern to malaria epidemiologists, indicating the urgent need to identify and develop alternate compounds capable of curing resistant malaria. Malaria pathogenicity can be diminished by administration of drugs which inhibit active multiplication of asexual erythrocytic parasites and cure can be achieved by effectively eliminating the blood forms from the host. The parasite clearance may be achieved either by lethal action of the compound used for treatment and/or by its
ability to evoke a stronger immune response against the parasite. Cisplatin has
twin properties of being employed for neoplastic treatment (Einhorn and Williams
1979; Rosenberg 1984) and immunomodulation (Page et al. 1977; Kleinerman and
Zwelling 1982). Cisplatin is a coordination compound of bivalent platinum and
was the first inorganic antineoplastic agent to enter into clinical investigations
around 1972 (Rosenberg 1973). Some of the known properties or salient features
of cisplatin have been enumerated as under:

1. The drug is active against a wide variety of tumors (Rosenberg 1973, 1980)
2. Inactivates transforming DNA and viruses (Munchausen 1974; Shooter
et al. 1972)
3. Induces filamentous growth and causes prophage induction in lysogenic
strains of E. coli bacteria (Rosenberg 1971, 1973)
4. The drug causes chromosomal abnormalities and toxicity (Vandenberg
and Roberts 1975)
5. Supresses graft rejection against H2 histocompatibility barrier in mice
(Khan et al. 1972)
6. Activates the immune system of the host (Rosenberg 1985)
7. Selectively inhibits DNA synthesis (Harder and Rosenberg 1970; Howley
and Gale 1970)
8. Causes mutagenesis (Monti-Bragdin et al. 1975; Lecointe et al. 1977)

To our knowledge no work has been reported on the effect of this compound
on malaria parasites. These considerations prompted us to assess antiplasmodial
properties of cisplatin in vitro and in vivo. Further, in order to understand the
mode of action of this compound, studies on stage-specific susceptibility of eryth-
rocytic parasites in vitro and interaction of cisplatin with intact or restriction en-
zyme digested P. falciparum DNA have been carried out. The later experiments
were performed with a view to look for changes, if any, in the number and size of restriction fragments generated, following incubation of \textit{P. falciparum} DNA with cisplatin, on a Southern blot using \textit{P. falciparum} specific oligonucleotide probe.

**MATERIALS AND METHODS**

**Parasites** Rodent malaria parasite \textit{P. berghei} was obtained from National Institute of Communicable Diseases, Delhi, India and maintained in Swiss albino mice of 30-40 g each, by weekly mechanical passage of infected erythrocytes drawn from a donor mouse by cardiac prick into a sterile, heparinized syringe. The biology of these parasites has been reviewed by Killick-Kendrik (1978). Chloroquine resistant \textit{P. falciparum} isolate FCD-4 of human origin which has been described in earlier section, was the other parasite used in this study.

**Stock solution of cisplatin** The stock solution of cis-platinum (II) diammine dichloride, obtained from Sigma Chemicals, USA, was prepared by dissolving 1 mg of the compound in 60% dimethylsulfoxide (DMSO). Further dilutions were made with buffered RPMI-1640 culture medium.

**Collection of parasitized blood from infected donor mouse**

The strain of \textit{P. berghei} was propagated in Swiss-albino mice by weekly mechanical passage of infected erythrocytes. The parasitized blood was drawn from an infected donor mouse via cardiac puncture. The infected blood was centrifuged at 2000 rpm for 10 min at 4 °C. The buffy coat and supernatant containing plasma were discarded. Packed cells were washed twice in 3.5 volumes of sterile 0.85% NaCl. This parasitized blood was used for passaging in mice and the course of infection followed by examining Giemsa stained blood films made each day from the tail blood.

**Inoculation of test animals**

Infected blood collected from the donor mouse usually contained about 70% parasitemia. The infected erythrocytes were washed with sterile normal saline as
described above. The number of cells per 0.05 ml of infected blood was calculated using Neubauer cell counting chamber. Percentage parasitemia was determined from Giemsa stained thin blood films. Each mice was inoculated with 0.05 ml of infected blood containing \(10^7\) to \(10^8\) parasites. Infected mice were kept in an air-conditioned room (24°C - 26°C). Mice were fed *ad libitum* with commercial pellet and water supplemented with 0.01% p-aminobenzoic acid.

**Erythrocytic schizontocidal activity**

**In vivo susceptibility of *P. berghei***: Curative property of cisplatin was assessed by comparing the survival times of mice untreated or treated with cisplatin receiving same amount of parasite inoculum in the form of infected erythrocytes obtained from a donor mouse. Test mice were taken in groups of five. Preliminary experiments were conducted in batches consisting of three mice each to arrive at the optimum tolerable dose of cisplatin effective in curing the mice. These preliminary doses ranged from 2-12 mg/kg body weight. Each member of the test group was injected with \(10^8\) parasitized erythrocytes, inoculated intraperitoneally. The cisplatin was given intraperitoneally but eight hours later either as a single dose or in four equally divided doses, at 4 hour intervals each in 1 ml saline solution. Appropriate sets of control mice were maintained. The development of parasitemia was monitored by making Giemsa stained blood films from the cut tail vein of the experimental and control animals every 24 hours for a period of 60 days.

**In vitro susceptibility of *P. falciparum***: Erythrocytic schizontocidal activity of cisplatin on *P. falciparum* was determined by exposing parasites in triplicates to graded concentrations of cisplatin using the modified 48 hour test method (Nguyen-Dinh and Payne 1980). The experiments were conducted in 24-well tissue culture plates by setting up microcultures with eight different concentrations, ranging from 1 to 1000 ng/ml. The parasite material for experiments was ob-
tained from stock cultures and subjected to sorbitol lysis (Lambros and Vanderberg 1979), to get synchronized ring stages. The parasitemia of the 50\% cell suspension of this synchronized material was adjusted to less than 1\% with uninfected freshly washed erythrocytes. Aliquot of 20 μl of the suspension was added into series of wells of the test plate, each holding 480 μl of complete medium with or without drug, yielding final cell suspension of 2\%. Loaded test plates were incubated at 37 °C in a candle-jar for 96 hours with daily change of medium. The drug was included in experimental wells for first two days only. Blood smears were made at the end of 48 and 96 hours, stained with Giemsa. Minimum of 5000 erythrocytes were enumerated to determine the parasitemia. Percentage reduction of parasitemia in relation to control was calculated. Fifty percent and 90\% inhibitory concentrations (IC_{50} and IC_{90}, respectively) were extrapolated from semi-log plot of varying concentrations of cisplatin against percentage inhibition of growth

**In vitro stage-specific sensitivity:**

To investigate the preferential toxicity to any specific stage of *P. falciparum* in vitro, asynchronous culture material was subjected to triple sorbitol treatment at 0, 40 and 47 hours interval, to obtain synchronized parasites of 0-7 hours old (Lambros and Vanderberg 1979). These synchronized parasites were in early ring stage for another 12 hours, for next 12-24 hours of development in late ring or early trophozoite stage, 24-36 hours old in late trophozoite stage and during subsequent 36-48 hours of growth in schizont stage of the erythrocytic schizogonic cycle. The synchronized stages were subjected to drug pressure for 12 hours each, the respective period of their residence in the specific stage. The experiments were conducted in triplicates in multiwell plates and slides made at the end of schizogonic cycle. The toxicity of cisplatin to ring, trophozoite and schizont infected stages was determined by calculating the percentage inhibition of parasitemia in drug treated parasites to that in drug free controls. The extent of syn-
chronization of parasites achieved is depicted elsewhere.

**Platination of DNA**

Binding of cisplatin to *P. falciparum* DNA, extracted from erythrocytic stages (Tungpradabkul and Panyim 1985) was studied by incubating 4 μg of DNA at 37 °C for 24 hours with 50 or 100 ng of cisplatin in total volume of 10 μl. The incubated material was then digested with restriction enzyme *Hind* III at 37 °C for 2 hours (Sambrook et al. 1989). In another set of experiment *Hind* III digested DNA material was incubated with cisplatin. Similar experiments were performed using *Taq* I. These digested samples were size fractionated on a 0.7% agarose gel along with control samples of uncleaved or restriction enzyme cleaved DNA not incubated with cisplatin. The gel was stained with ethidium bromide and visualized under UV before the material was transferred on to a nylon membrane by Southern blotting. The membrane was then hybridized with γ-32P end labelled 21-mer synthetic oligonucleotide, which is known to be a repetitive sequence in *P. falciparum* genome (Mucenski et al. 1986), at 42 °C using stringent washing conditions. The methods employed for the extraction of DNA, Southern transfer and hybridization have been described in the following chapters. The sequence of the oligomer used to probe the membrane was as follows:

5'-AGGTCTTAACTTGACTAACAT-3'

**RESULTS**

The periodicity of blood schizogony of the rodent malaria parasite *P. berghei* was a quotidian cycle i.e., 22-25 hours. The mortality rate of *P. berghei* in the strain of white mice used in the present study was 100%, if untreated. The mortality curve was bimodal. During the first 6 days of infection, mature erythrocytes were invaded, and the mouse died in a condition of shock. If the animal survived the first phase, many reticulocytes then occurred in the blood and were preferen-
tially invaded, anemia increased and the animal died later in a condition of anoxia. Parasites were observed in tail blood films of test animals within 24 hours after inoculation of infected blood. As seen in Figure 4.1 a & b the characteristic course of infection was highly asynchronous. Blood films showed the presence of all the different asexual blood stages of the parasite namely rings, trophozoites and schizonts at the same time. Reticulocytes were frequently infected with multiple parasites than mature erythrocytes. Upto 10 parasites per reticulocyte were observed. The intraerythrocytic ring forms showed red staining chromatin dot with a very small area of blue stained cytoplasm. The trophozoites were recognizable by the presence of chromatin dot with enlarged cytoplasm containing faint brown-yellowish spots of haematin granules. Mature schizonts were characterized by 12-20 distinct merozoites and a dark brown-yellowish dot of clustered haematin. These schizonts in due course became more fragile and released their merozoites, resulting in groups of free merozoites accompanied by a free pigment cluster (Figure 4.1 b). Platelets and white blood corpuscles are seen in Figure 4.2.

Ability of cisplatin to clear *P. berghei* infection from mice is presented in Table 4. Total dose of 6 mg/kg body weight given in four equally divided intraperitoneal injections at four hour intervals effectively cured the infected mice and no parasitemia could be observed for sixty days in any of the treated mice. The same dose when given as a single intraperitoneal injection finally cured 80% of the infected mice. Initially, however, in three out of five of these test animals parasite appearance was delayed by 11 days compared to controls and parasite population increased subsequently leading to death of one of the mice whereas in the other two mice appearance of high parasitemia was followed by slow decline with final disappearance of the parasites after 14 days. Four week onwards from the start of the experiment no parasite could be observed in these two surviving mice till day sixty. In all the control animals, not receiving treatment with cisplatin, healthy parasites appeared in circulation within 24 hours of receiving the infective inocu-
lum and they died in less than 10 days due to high parasitemia.

Table 4.1 Ability of cisplatin to cure *P. berghei* infected mice. Each of the mice received an intraperitoneal innoculum of $1 \times 10^8$ parasites. The treated animals were injected intraperitoneally with cisplatin 8 hr later.

<table>
<thead>
<tr>
<th>Infected mice</th>
<th>Untreated</th>
<th>Treated with cisplatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose</td>
<td>nil</td>
<td>6mg/kg body weight</td>
</tr>
<tr>
<td></td>
<td></td>
<td>as a single dose</td>
</tr>
<tr>
<td>Mice showing</td>
<td>5/5</td>
<td>3/5</td>
</tr>
<tr>
<td>parasitemia</td>
<td></td>
<td>0/5</td>
</tr>
<tr>
<td>within two</td>
<td>0/5</td>
<td>4/5</td>
</tr>
<tr>
<td>weeks of</td>
<td></td>
<td>5/5</td>
</tr>
<tr>
<td>innoculation</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Inhibitory effect of cisplatin on multiplication of the synchronized cultures of *P. falciparum* in vitro are presented in Figure 4.3. Complete inhibition of the multiplication was achieved by minimum amount of 30 ng/ml of cisplatin present in culture medium for a duration of 48 hours. From the dose response curve IC$_{50}$ and IC$_{90}$ values determined after 48 hours were found to be 6.2 and 24 ng/ml, respectively and 3.8 and 26 ng/ml, respectively after 96 hours experiment. Abnormal parasites were observed from drug treated wells at the end of 48 hours, showing different degree of vacuolization or pale staining, whereas normal healthy parasites multiplied to about six fold in control wells in a schizogonic cycle. Most of the abnormal parasites in treated wells had degenerated or lysed by 96 hours.
The stage specific toxicity of cisplatin is given in Figure 4.4. Schizont stages were least susceptible to the toxic effect of the compound followed by young rings whereas trophozoites and old rings were most susceptible. The schizont stages exposed to 30 ng/ml of cisplatin in the medium resulted in only 36% growth inhibition.

Autoradiographs of the restriction enzyme digested falciparum DNA incubated with or without cisplatin and probed with a synthetic repetitive oligonucleotide are shown in Figures 4.5 & 4.6. No hybridization band was observed in the lanes containing uncut DNA or digested DNA incubated with cisplatin whereas control lanes showed the clear presence of one or several hybridization bands.

DISCUSSION

One of the most inexpensive ways of searching new lead antimalarial compounds for drug development could be to screen the drugs which have been developed for other diseases and evaluate their antimalarial potentials, either alone or in combination with other known antimalarial drugs. Cisplatin is a drug with high potency in treatment of a few human malignancies (Rosenberg 1985; Hosokawa et al. 1982; Volge et al. 1982) and is a broad spectrum anticancer drug in animals (Rosenberg 1985; Welsh 1971). It is believed that the drug basically enhances the immunogenicity of the tumor cells by exposing new antigenic sites on the tumor cell surface and this evokes a host of offending immune reactions directed against the tumors. This assumption is based on the following observations: a) Swiss white mice cured of advanced Sarcoma-180 tumors with therapeutic dose of cisplatin completely rejected all further transplants for 11 subsequent months because of high level of immunity generated against the tumor lasting for this period (Rosenberg 1973, 1985). b) In the Swiss white mice cisplatin treatment produced nearly absolute cure of advanced Sarcoma-180 tumors with a single intraperitoneal injection on day eight but administration of hydrocortisone, an
immunodepressed, on a day prior to the injection of cisplatin produces only 40% cure by eighth day (Conran and Rosenberg 1972). *P. falciparum* also possesses genes with affinities to multiple drug-resistance (mdr) genes of human cancer cells (Bradely et al. 1988; Foote et al. 1989) and this particular factor provided a strong clue that some anticancer drugs may possess antiplasmodial properties. The results presented in this report amply demonstrate the antiplasmodial potential of cisplatin. Apparently non-pathogenic dose of this compound cured the malaria infected mice and in vitro inhibited multiplication of a chloroquine resistant falciparum isolate at a low concentration included in the medium.

Toxicity to the host is an important consideration of any candidate compound with potential of being employed as a drug. The amount of cisplatin used in this study to cure malaria infection in mice is well tolerated by the host. The dose of 6 mg/kg body weight given either as a single intraperitoneal push-in injection or in four equally divided doses at 4 hour intervals with saline did not cause any mortality in control mice. Other cancer workers have also reported that therapeutic dose of 7 mg/kg body weight of cisplatin causes less than 10% mortality in mice and as a single intraperitoneal injection, the administered LD$_{50}$ dose is 13 to 14 mg of cisplatin/kg body weight (Rosenberg 1985). Slow infusion of cisplatin gives better results in cancer treatments (Rosenberg 1985) and also kidney toxicity is ameliorated with saline hydration (Hayes et al. 1977). Present report also shows that 1.5 mg/kg body weight dose of cisplatin 4 times at 4 hour intervals with saline is far more superior than a single dose of 6 mg/kg in treatment of mice malaria. This is perhaps due to longer retention of higher level of cisplatin in the body for extended period of time, as half life of cisplatin in mice has been reported to be only 20 hours and an erythrocytic schizogonic cycle of mice malaria is of 24 hour duration. In the two mice, which received single 6 mg/kg body weight dose of cisplatin, appearance of parasitemia was followed by slow but complete recovery, this could be due to immunostimulation property of cisplatin which has been clearly
demonstrated in several reports (Kleinerman et al. 1980; Kleinerman and Zwelling 1982; Bahadur et al. 1984). Cisplatin has trypanocidal activity in mice (Wysor 1982). The preliminary result in this study clearly demonstrates the antiplasmodial properties of cisplatin both in vitro and in vivo systems. Therefore, it would be worthwhile to investigate the antimalarial properties of this and other related platinum complexes with regard to their ability to cure polyresistant Plasmodium species. Probing additivity and synergism properties in combination with other antimalarial drugs having different mode of actions should also not be ignored, but all this needs further serious investigations.

Amount of cisplatin required in vitro to achieve 50% inhibition of parasitemia in isolate FCD-4 was found to be less than either chloroquine (IC50 = 18 ng/ml, our unpublished data) or quinine (IC50 = 11 ng/ml) (Nair and Bhasin 1993). Not much difference in IC50 and IC90 values calculated from the slides made at the end of 48 or 96 hours of the experiment suggests that there was an insignificant delayed mortality of the parasites due to cisplatin toxicity. Most of its lethal effect is exhibited within an erythrocytic schizogonic cycle.

Each of the synchronized asexual erythrocytic stages were subjected in vitro to uniform drug pressure of 12 hours with differential outcome with regard to parasite survival and multiplication. Intraerythrocytic merozoites in the schizont and the early rings are the least susceptible to toxic effects of cisplatin, perhaps in these stages no active DNA synthesis takes place (Inselburg and Banyal 1984) and the parasite plasma membrane is most intact thus retarding free entry of the compound into the parasite milieu. Cisplatin is known to prevent the multiplication of cancer cells by randomly inter- and intralinking of DNA strands of these dividing cells (Cohen et al. 1979). The most damaging outcome, therefore, are seen in the early and late trophozoite stages of malaria parasite, known to be actively involved in DNA synthesis (Inselburg and Banyal 1984).

Molecular basis of cisplatin toxicity has been attributed to lesions in DNA
molecules of cancer cells leading to inter- and intrastrand DNA cross-links, fi-
nally resulting in cell death (Cohen et al 1979; Zwelling and Kohn 1979). In vitro
binding of cisplatin can occur with any base but prefered order of binding is
G>A>C>T (Rahn et al. 1980). We explored the interaction of cisplatin with P.
falciparum DNA, by incubating it with intact DNA and digesting it later with
restriction enzymes and by incubating predigested DNA with cisplatin. When these
samples were run on agarose gel and stained with ethidium bromide, no fluo­
rescence was observed in lanes containing samples incubated with cisplatin, showing
that cisplatin prevents intercalation of ethidium bromide with DNA. Following
blotting of these samples on to the nylon membrane and on probing with radiola-
belled 21-mer repeat sequence, no hybridization in these lanes are visible. This
may be due to inter- and intrastrand cross linking of DNA by cisplatin, thus pre­
venting the specific binding of the probe to complementary sequences on the DNA
which is clearly seen in the form of distinct bands in control lanes of the autorad­
iograph. Thus mode of action of this compound on Plasmodium may be similar to
the reported mechanism of action on neoplastic cells.

Figure 4.1 a. A Giemsa stained thin blood film from *P. berghei* infected mouse showing different asexual stages of the parasite namely ring (R), trophozoite (T) and schizont (S).

Figure 4.1 b. A Giemsa stained thin blood film from *P. berghei* infected mouse showing multiple infection of reticulocytes, a cluster of free merozoites and pigment.
Figure 4.2  A Giemsa stained thin blood film from *P. berghei* infected mouse showing parasites, platelets (P) and WBC (W).
Figure 4.3 In vitro growth inhibition response of asexual erythrocytic stages of *P. falciparum* to different concentrations of cisplatin in 48 hour test. Slides were made at the end of 48 and 96 hours. Starting synchronized parasitemia was less than 1%.
Figure 4.4  In vitro growth inhibition of different asexual erythrocytic stages of *P. falciparum* to various concentrations in a 48 hour test. Slides were made at the end of 48 hours. Starting parasitemia in each case was less than 1%.
Figure 4.5  An autoradiogram showing hybridization bands of *P. falciparum* DNA with species-specific synthetic $\gamma^{32}$P labelled 21-mer oligonucleotide probe. The DNA samples (4 µg in each lane except lane 3, which was blank) were size fractionated on 0.7% agarose gel and Southern transferred on to nylon membrane for hybridization.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Undigested <em>P. falciparum</em> DNA</td>
</tr>
<tr>
<td>2.</td>
<td>DNA digested with <em>Hind III</em></td>
</tr>
<tr>
<td>3.</td>
<td>Kept blank</td>
</tr>
<tr>
<td>4.</td>
<td>DNA incubated with DMSO prior to digestion with <em>Hind III</em></td>
</tr>
<tr>
<td>5.</td>
<td>DNA digested with <em>Hind III</em> and incubated with DMSO</td>
</tr>
<tr>
<td>6.</td>
<td>DNA incubated with 50 ng of cisplatin</td>
</tr>
<tr>
<td>7.</td>
<td>DNA digested with <em>Hind III</em> and incubated with 50 ng of cisplatin</td>
</tr>
<tr>
<td>8.</td>
<td>DNA incubated with 100 ng of cisplatin and later digested with <em>Hind III</em></td>
</tr>
<tr>
<td>9.</td>
<td>DNA digested with <em>Hind III</em> and incubated with 100 ng of cisplatin</td>
</tr>
</tbody>
</table>
Figure 4.6 An autoradiogram showing hybridization bands of *P. falciparum* DNA with species-specific synthetic $\gamma^{32}$P labelled 21-mer oligonucleotide probe. The DNA samples (4 μg in each lane except lane 3, which was blank) were size fractionated on 0.7% agarose gel and Southern transferred on to nylon membrane for hybridization.

Lane 1. *P. falciparum* DNA incubated with 50 ng of cisplatin and then digested and then digested with *Taq I*
2. DNA digested with *Taq I*
3. DNA digested with *Taq I* and then incubated with 50 ng of cisplatin
4. DNA incubated with DMSO prior to digestion with *Taq I*
Section - II
Chapter 5

IN VITRO SELECTION OF P. FALCIPARUM RESISTANT LINES TO DIHYDROFOLATE-REDUCTASE INHIBITORS, CROSS RESISTANCE AND OTHER RELATED STUDIES

Abstract: Proguanil and pyrimethamine exhibit their antimalariaIs 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. 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.

INTRODUCTION

Pyrimethamine and cycloguanil, the active metabolite of proguanil, are the two most widely used DHFR inhibitor antimalarial drugs. Soon after introduction of proguanil, reports of resistance in P. falciparum started emanating from endemic areas around the world (Chaudhuri and Chaudhuri 1949; Field and Edeson 1949; Adams and Seaton 1949; Covell et al. 1949). These observations led Nicol (1953) to suggest that 200 mg daily dose of proguanil would be better than 100 mg. Since that time there have been numerous reports of the failure of daily proguanil prophylaxis (100 mg) to prevent the development of P. falciparum, but with very few well documented case histories. The real incidence of proguanil prophylactic resistance appears to be in doubt, but many British authorities retain a firm belief in continuing efficacy of this compound as a prophylactic against falciparum malaria (Peters 1984a). Proguanil is not available and used in India since early 1950s. This is mainly because of the availability of pyrimethamine which has
proved, weight for weight, to be many times more active than proguanil (Bruce-Chwatt et al. 1981), less expensive and has a longer retention time, thus it superseded the use of proguanil. Pyrimethamine resistance has followed a similar history to that against proguanil. Since 1952 there have been many reports of pyrimethamine resistance in all species of human malaria (Coatney et al. 1952; Wilson 1952; Young 1957; Burgess and Young 1959). There is abundant evidence to indicate that pyrimethamine resistance is widely distributed throughout malaria endemic areas of the world (WHO 1965, 1973), so also in India (Gajanana et al. 1984). It will be of interest to ascertain the susceptibility of pyrimethamine resistant and sensitive lines from an Indian isolate to cycloguanil in vitro, the drug which has not been in wide use for over fifty years now in India and in the absence of any authentic clinical data from other endemic areas of the world with regard to susceptibility to cycloguanil. These observations/considerations provided an impetus to design the following experiments with defined objectives, with a view to assess the potential usefulness of cycloguanil in circumventing and combating the problem of pyrimethamine-resistance in falciparum:

a) To evaluate the speed of selection of resistance to the two DHFR inhibitor antimalarial drugs by subjecting a cloned *P. falciparum* line in vitro to pyrimethamine or cycloguanil pressure. b) To isolate a series of progressively resistant *P. falciparum* lines to each of these two drugs that will be useful in the study of the mechanisms of evolution of resistance to DHFR inhibitors. c) To determine the extent of cross-resistance acquired to each of them at various stages of resistance and to look susceptibility profile changes, if any, to other antimalarials. d) To ascertain if resistance to one drug predisposes the parasites to be selected faster for resistance to the other drug. e) The two DHFR inhibitors are toxic to the same or different stages of *P. falciparum*. f) Synergistic or additive virtues of these two compounds in vitro. g) To characterize the resistant lines selected with regard to biological advantage, if any.
This chapter describes the findings of aforementioned experiments.

MATERIALS AND METHODS

Parasites

Isolate FCD-4 and a clone F-56 derived from this isolate were the two *P. falciparum* parasite lines used in the present study. The clone was obtained by limiting dilution method (Rosario 1981) from the erythrocytic stages of parasites cultivated continuously in vitro employing candle-jar procedure of Trager and Jensen (1976). The FCD-4 was isolated locally from an infected patient (Nair and Bhasin 1993).

Stock solutions of drugs

Pyrimethamine $10^{-2}$ M stock solution was prepared in 0.05% lactic acid and $10^{-2}$ M stock of cycloguanil in 70% ethanol. Chloroquine sulphate solution contained chloroquine base equivalent to 40 mg /ml. Methotrexate (amethopterin) $10^{-2}$ M stock solution was prepared in 60% DMSO. Artemisinin (qinghaosu) 1mg/ml of the stock solution was made in DMSO. Drugs were filter sterilized and stored at 4 °C. Further dilutions were made in RPMI-1640 medium prior to use.

Stock culture of parasites

Parasite stocks were maintained in glass Petridishes (10 cm diameter) by the candle jar method (Trager and Jensen 1976) in A+ human erythrocytes using RPMI-1640 medium supplemented with AB+ serum. Blood products were procured commercially. Starting parasitemia in these cultures was less than 1% and hematocrit 8% in a total of 12 ml erythrocyte cell suspension in each Petridish. The cultures were diluted every 4 or 5 day with uninfected erythrocytes to restore the parasitemia and hematocrit to less than 1% and 8%, respectively.

In vitro Gametocytogenesis

Cultures were initiated in glass Petridishes to determine the capability of falciparum isolate/clone to form gametocytes in vitro (Bhasin and Trager 1984).
Each Petridish (5 cm diameter) was seeded with 5 ml of culture material having 8% hematocrit and less than 1% parasitemia. An initial zero hour slide of seeding material was made to determine the exact parasitemia and gametocytemia. The cultures were incubated at 37 °C in a candle-jar with daily change of medium for eight consecutive schizogonic cycles without addition of uninfected erythrocytes. Thick blood smear of 10 μl material of 50% cell suspension was made every 48 hours. Gametocytemia was assessed by examining minimum of 100 fields. The gametocytes were classified as stage II to V (Hawking et al. 1971).

Susceptibility test method

Susceptibility of parasites to DHFR inhibitors and other antimalarials was determined by exposing parasites in triplicates to graded concentrations of each drug using the modified 48 hours test method (Nguyen-Dinh and Payne 1980). The experiments were conducted in 24-well tissue culture plates by setting up microcultures with different drug concentrations (ranging from 10^{-11} to 10^{-8} M with five fold difference or less for DHFR inhibitors). The parasite material for experiments was obtained from stock cultures and subjected to sorbitol lysis (Lambros and Vanderberg 1979), to obtain synchronized ring stages. The parasitemia was adjusted to less than 1% with uninfected fresh erythrocytes and this material was made to 50% cell suspension with complete RPMI medium. Aliquots of 20 μl of the suspension were added into series of wells of the test plate, each holding 480 μl of complete medium with or without drug, yielding final cell suspension of 2%. Loaded test plates were incubated at 37 °C in a candle-jar for 96 hour with daily change of medium. The drug was included in experimental wells for first two days only. Blood smears were made at the end of 48 and 96 hours, stained with Giemsa. Minimum of 5000 erythrocytes were enumerated to determine parasitemia in each well. Percentage reduction of parasitemia in relation to control was calculated from percentage inhibition of growth. Fifty percent, 90% and minimum complete inhibitory concentration (IC_{50}, IC_{90} and MIC, respectively
were extrapolated from semilog plot of varying concentrations against percent inhibition of growth obtained from triplicate wells.

Selection for increased antifol resistance

The cloned falciparum line was subjected in vitro to several rounds of increasing drug pressure in multistep manner to select a series of progressively resistant P. falciparum lines. Small glass Petridishes holding 5 ml of drugged medium with starting erythrocytic cell suspension of 5% hematocrit and 2% parasitemia were incubated at 37 °C for 48 hour, drugged medium was replenished after 24 hours. The drug concentration selected for exposure of the parasites, in the first-step, was little lower than the IC₅₀ of the parasites. The surviving parasites were allowed to multiply in the drug free medium till the parasitemia reached to about 2%, and these parasites were re-exposed to the first-step drug concentration for another 48 hours followed by growth in drug free medium. This procedure continued till the 48 hours drug pressure concentration used in the first-step did not retard the parasite multiplication, that is atleast five fold increase in parasitemia could be obtained in a schizogonic cycle with starting parasitemia of around 0.5%. In the second-step these parasites were exposed for 96 hours to the selected concentration of the drug, followed by the propagation in the drug free medium until the parasitemia reached to around 2%. Drug was included again in the medium for 96 hours duration of parasite multiplication and the procedure was repeated till atleast five fold increase in parasitemia was achieved in the first 48 hours with starting parasitemia of around 0.5% in presence of drug pressure. In the final-step of the first round of selection the surviving parasites were grown under continuous drug pressure till the rate of multiplication of these parasites was equal to that of control parasites grown in drug free medium, for a few cycles. The selected parasites of first round of selection were subjected to next round of selection pressure with five fold (or less) increase in the drug concentration by the same multistep procedure as described for the first round. This protocol for selec-
tion was repeated for each of the subsequent rounds. Selected parasites were peri-
odically cryopreserved (Rowe et al. 1968) in liquid nitrogen for future use.

Cross resistance

The derived falciparum line resistant to one of the DHFR inhibitors was
evaluated for its sensitivity profile to the other antifol. In addition the susceptibility
of the highly pyrimethamine-resistant line to other antimalarials and another
DHFR inhibitor amethopterin was evaluated to determine the degree of cross re-
sistance acquired by this selected line.

Stability of resistance

In order to assess, if the selected resistant phenotype of the parasite cell
line is stable or lost once the drug pressure is discontinued for an extended period
of time during continuous cultivation, the susceptibility to pyrimethamine of these
parasites was determined at the end of 60 weeks of continued growth in drug free
medium and compared with that of the original resistant cell line kept under
constant drug pressure. The parasites were cultivated in glass Petridishes hold-
ing 5 ml of erythrocyte suspension in RPMI-1640 by candle jar method, with
dilution of cultures every 4 or 5th day by restoring the parasitemia to less than 1%
and hematocrit to 5%.

Stage-specific sensitivity to DHFR inhibitors

Highly synchronized parasites of clone F-56 were employed to look for dif-
fferences in toxicity of pyrimethamine and cycloguanil, if any, to the specific stages
of P. falciparum. Asynchronously multiplying parasites with atleast 5% parasitemia
and containing majority of the parasites in the ring stage were used for triple
sorbitol synchronization (Lambros and Vanderberg 1979). The culture material
was centrifuged at 2000 rpm for 5 min, the supernatant was discarded and the
pellet resuspended in 5 volumes of sterile 5% sorbitol in distilled water for 7 min
at room temperature. After incubation the parasite material was centrifuged and
the supernatant discarded. The pellet was washed thrice with RPMI medium and
culture was reestablished by addition of fresh uninfected erythrocytes in the medium containing 10% serum supplement. At 40 hours following the first sorbitol treatment the parasites were treated again with 5% sorbitol in the same aforementioned manner to increase the synchronization (Vial et al. 1982). Freshly washed erythrocytes were added to the infected cells and culture was set again. Seven hours later the culture material was subjected to third and final treatment with sorbitol, so as to obtain 0-7 hour old ring stage parasites. The pellet was thoroughly washed in order to avoid the influence of trace amounts of sorbitol on the parasites. This material was mixed with freshly washed uninfected cells to obtain the final parasitemia between 0.5 to 1% in 50% erythrocyte cell suspension.

Stage-specific effect of pyrimethamine and cycloguanil was determined by exposing different synchronized erythrocytic stages of *P. falciparum* clone F-56 to either $1.5 \times 10^{-9}$ M concentration of pyrimethamine or $7.2 \times 10^{-11}$ M cycloguanil, the concentrations used cause 50% inhibition of parasite growth in vitro. The experiments were performed in 24-well tissue culture plates. Infected erythrocyte material in aliquots of 20 µl with about 0.7% parasitemia was inoculated in each well. The experiment was divided into following five test groups:

Group 1- as control group, parasites were cultivated in drug free medium

Group 2- early ring stage, parasites were cultured in medium containing either cycloguanil or pyrimethamine from 0-12 hours and thereafter in drug free medium.

Group 3- parasites were exposed to the drug in the late ring or early trophozoite stage that is the parasites were cultured in medium containing drug from 12-24 hours.

Group 4 - parasites were exposed to the drug in trophozoite stage of their life cycle that is they were cultured in the presence of drug from 24 to 36 hours of the 48 hours of life cycle.

Group 5 - parasites were cultured in medium containing drug in the schizont stage,
the parasites were kept in drug free medium from 0-36 hours followed by 12 hours in presence of the drug.

The experiments were done in triplicate. At the end of 48 hours thin blood films were made from each of the experimental wells. The slides were stained in Giemsa solution. Antiparasite effect of the drugs on early rings, late rings, trophozoite and schizont infected erythrocytes were determined by comparing the percentage inhibition of parasite growth in drug treated parasites to that in drug free controls.

**Synergism**

Synergy, the ability of one drug to potentiate the activity of another drug was evaluated algebraically by determining the sum of the fractional inhibitory concentration (FIC) of a combination of drugs. The FIC of a drug is its effective concentration in combination with another drug divided by its effective concentration alone. When the sum of the FIC is < 1; it indicates synergism; value = 1 indicates additive effect and values > 1 indicates antagonism. To examine the combined effect of pyrimethamine and cycloguanil in vitro on pyrimethamine resistant line derived from the clone F-56 the method of Berenbaum (1978) was employed. First IC<sub>90</sub> values for each drug when used alone was determined. The parasites were exposed for this purpose either to different concentrations ranging from 1 x 10^{-8} M to 8 x 10^{-8} M of pyrimethamine or 6 x 10^{-5} M to 4 x 10^{-7} M of cycloguanil in multiwell plates for 48 hours and IC<sub>90</sub> values were determined by studying the slides made at the end of 96 hours, as described elsewhere. To test for synergism, a reference combination of the two drugs consisting of half of the concentration close to IC<sub>90</sub> of each drug was prepared in complete RPMI medium and actual IC<sub>90</sub> of this combination was determined together with parallel determination of IC<sub>90</sub> values of the drugs when used alone for estimating the FIC values.

**Biological advantage of pyrimethamine resistant parasites**
In order to assess any biological advantage acquired by the pyrimethamine resistant (PS-5) line selected over the parent sensitive clone F-56 with regard to growth in vitro, the following two experiments were performed. First, the growth rate of the sensitive and the resistant parasites was compared in vitro. Second, equal number of PS-5 and F-56 parasites were mixed, and sensitivity to pyrimethamine of this mixed population evaluated immediately and after a year of continuous cultivation in drug free medium. Aliquots of the mixed parasite culture prepared at the beginning of experiment was also cryopreserved for subsequent resuscitation and comparison of pyrimethamine sensitivity with that of the mixed parasite population kept under continuous cultivation for a year.

RESULTS

Susceptibility profile of the parasites used: Susceptibility of *P. falciparum* clone F-56 and the isolate FCD-4 to pyrimethamine, cycloguanil, and chloroquine was determined in vitro. *IC_{50} and IC_{90} values are presented in Table 5.1.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Clone F-56</th>
<th>Isolate FCD-4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC_{50}</td>
<td>IC_{90}</td>
</tr>
<tr>
<td>Pyrimethamine*</td>
<td>1.5</td>
<td>3.6</td>
</tr>
<tr>
<td>Cycloguanil*</td>
<td>0.072</td>
<td>0.27</td>
</tr>
<tr>
<td>Chloroquine*</td>
<td>46.89</td>
<td>84.4</td>
</tr>
</tbody>
</table>

Data in *= nM
MIC for each of these drugs respectively was found to be $5 \times 10^{-8} \text{M}$, $5 \times 10^{-10} \text{M}$ and $1.87 \times 10^{-7} \text{M}$ for clone F-56. MIC required for FCD-4 for pyrimethamine, cycloguanil, and chloroquine was $10^{-8} \text{M}$, $4 \times 10^{-9} \text{M}$ and $1.87 \times 10^{-7} \text{M}$, respectively. Chloroquine was found to be equitoxic to the clone and the parental isolate as observed by MIC.

**In vitro gametocytogenesis**

Isolate FCD-4 and the clone F-56 were used for gametocytogenesis. Thick smear of the isolate made at the end of first schizogonic cycle showed the presence of a few gametocytes. The number of gametocytes in the thick smears increased in the subsequent schizogonic cycles. However, no gametocyte formation was observed in any of the smears made from the clone F-56 maintained under similar conditions for eight schizogonic cycles. The F-56 was confirmed to be non-gametocyte forming clone under in vitro conditions of continuous cultivation in drug free medium by performing these experiments repeatedly. The gametocytes were not observed even after 20 days of continuous culture without dilution.

**Selection of falciparum lines resistant to pyrimethamine**

The number of days required to select in vitro a series of increasingly pyrimethamine resistant *P. falciparum* lines from the clone F-56 and the drug concentrations used in each cycle of the selection process are depicted in Figure 5.1A. It took a total of 348 days of continuous cultivation of F-56, in presence or absence of drug pressure, to obtain the most resistant parasites that could multiply at the highest selective drug concentration of pyrimethamine ($10^{-8} \text{M}$) employed. In total of seven rounds of selection there was five or twofold increase of drug pressure during each successive rounds. A series of progressively resistant cell lines from PS-1 to PS-5 were cryopreserved in between and their sensitivity to
pyrimethamine in vitro evaluated, shown in Table 5.2. Each successive line of the series was less sensitive to the preceding line by 2.4 to 8 folds as adjudged by IC_{50} value. The parasite line PS-5 showed 2400 fold decrease in sensitivity to pyrimethamine compared to parent clone F-56.

**Table 5.2** Sensitivity to pyrimethamine of the series of selected parasite lines derived in vitro.

<table>
<thead>
<tr>
<th>Selected cell line</th>
<th>IC_{50}</th>
<th>IC_{90}</th>
<th>MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-56 clone</td>
<td>1.5 x 10^{-9} M</td>
<td>3.6 x 10^{-9} M</td>
<td>5 x 10^{-9} M</td>
</tr>
<tr>
<td>PS-1</td>
<td>3.8 x 10^{-9} M</td>
<td>2.3 x 10^{-8} M</td>
<td>5 x 10^{-8} M</td>
</tr>
<tr>
<td>PS-2</td>
<td>2.6 x 10^{-8} M</td>
<td>9.4 x 10^{-8} M</td>
<td>5 x 10^{-7} M</td>
</tr>
<tr>
<td>PS-3</td>
<td>2.1 x 10^{-7} M</td>
<td>8 x 10^{-7} M</td>
<td>2.7 x 10^{-6} M</td>
</tr>
<tr>
<td>PS-4</td>
<td>5.1 x 10^{-7} M</td>
<td>6.2 x 10^{-6} M</td>
<td>5 x 10^{-6} M</td>
</tr>
<tr>
<td>PS-5</td>
<td>3.6 x 10^{-8} M</td>
<td>8 x 10^{-8} M</td>
<td>&gt; 1 x 10^{-5} M</td>
</tr>
</tbody>
</table>

**Selection of falciparum line resistant to cycloguanil**

To select cycloguanil resistant falciparum lines the clone F-56 was continuously cultivated in vitro with or without cycloguanil pressure for a total duration of 351 days. Only about seventy five fold decrease in sensitivity was registered in the derived line during this selection. Time required to achieve each level of decreased sensitivity to cycloguanil is shown in Figure 5.1 B. In vitro sensitivity studies with cycloguanil showed that IC_{50}, IC_{90} and MIC of the parasite line derived at the end of selection period to cycloguanil were 5.4 x 10^{-9} M, 3.6 x 10^{-8} M
and 5 x 10^{-8} M, respectively.

**Gametocytogenesis during selection**

An interesting observation made during the selection process was the formation of gametocytes in culture by the non-gametocyte forming clone F-56 under drug pressure. Healthy gametocytes of various stages were observed in thick and thin blood films made from the cultures kept under sub-inhibitory concentrations of the drugs. At concentrations equal to or higher than the MIC the gametocytes formed were phenotypically unhealthy. Gametocyte production was very low, since in most of the blood films examined less than one gametocyte per 15,000 erythrocytes was observed. No correlation could be made between the concentration of drugs in culture and the number of gametocytes formed.

**Cross resistance**

The falciparum lines selected for pyrimethamine resistance in vitro acquire a degree of cross resistance to cycloguanil and to another potent DHFR inhibitor, amethopterin (methotrexate). Results of the cross resistance studies to these drugs are presented in Table 5.3 and 5.4.

**Table 5.3** Sensitivity to cycloguanil of the series of pyrimethamine selected parasite lines derived in vitro.

<table>
<thead>
<tr>
<th>Selected cell line</th>
<th>IC_{50}</th>
<th>IC_{90}</th>
<th>MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-56 clone</td>
<td>7.2 x 10^{-11} M</td>
<td>2.7 x 10^{-10} M</td>
<td>5 x 10^{-10} M</td>
</tr>
<tr>
<td>PS-1</td>
<td>6.2 x 10^{-11} M</td>
<td>4.9 x 10^{-10} M</td>
<td>1 x 10^{-9} M</td>
</tr>
<tr>
<td>PS-2</td>
<td>4.6 x 10^{-11} M</td>
<td>6 x 10^{-10} M</td>
<td>3.8 x 10^{-9} M</td>
</tr>
<tr>
<td>PS-3</td>
<td>3 x 10^{-9} M</td>
<td>8.6 x 10^{-9} M</td>
<td>5 x 10^{-8} M</td>
</tr>
<tr>
<td>PS-4</td>
<td>7 x 10^{-9} M</td>
<td>4.3 x 10^{-8} M</td>
<td>8 x 10^{-8} M</td>
</tr>
<tr>
<td>PS-5</td>
<td>7.4 x 10^{-8} M</td>
<td>3.1 x 10^{-7} M</td>
<td>4 x 10^{-7} M</td>
</tr>
</tbody>
</table>
Table 5.4  Sensitivity of clone F-56 and the highly pyrimethamine-resistant selected parasite line PS-5 to chloroquine (CQ), artemisinin (QHS) and amethopterin (MTX) in vitro.

<table>
<thead>
<tr>
<th>Drug</th>
<th>F-56</th>
<th></th>
<th>PS-5</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>IC&lt;sub&gt;90&lt;/sub&gt;</td>
<td>MIC</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
</tr>
<tr>
<td>CQ*</td>
<td>0.011</td>
<td>0.027</td>
<td>0.06</td>
<td>0.017</td>
</tr>
<tr>
<td>QHS*</td>
<td>0.0017</td>
<td>0.004</td>
<td>0.005</td>
<td>0.0015</td>
</tr>
<tr>
<td>MTX</td>
<td>2.9x10&lt;sup&gt;-8&lt;/sup&gt;M</td>
<td>6.9x10&lt;sup&gt;-8&lt;/sup&gt;M</td>
<td>1x10&lt;sup&gt;-7&lt;/sup&gt;M</td>
<td>1.5x10&lt;sup&gt;-7&lt;/sup&gt;M</td>
</tr>
</tbody>
</table>

* values in µg/ml

Selection of cycloguanil resistance in pyrimethamine resistant falciparum derived line (PS-5)

Predisposition, if any, of the pyrimethamine resistant cell line to be selected faster for cycloguanil resistance was evaluated by exposing highly resistant PS-5 line to increasing concentrations of cycloguanil in a step wise fashion in vitro for 40 days. During this short duration of selection there was 7.3 fold decrease in the sensitivity to cycloguanil.

Stability of resistance

There was no marked difference observed in the susceptibility to pyrimethamine of PS-5 parasite line, determined at the start and end of sixty weeks of continuous cultivation without drug pressure. IC<sub>50</sub>, IC<sub>90</sub> of this line at the beginning and at the end of this experiment were found to be 3.6 x 10<sup>-6</sup>M, 8.8 x 10<sup>-6</sup>M and 4.1 x 10<sup>-6</sup>M, 8.2 x 10<sup>-6</sup>M, respectively.

Stage-specific sensitivity to DHFR inhibitors

The experimental parasite material examined immediately after triple sor-
bitol treatment consisted almost entirely of single and multiple ring form infections and uninfected erythrocytes. The synchronised parasite stages obtained are shown in Figures 5.2 to 5.5. No significant lysis of uninfected erythrocytes was observed.

The IC$_{50}$ value of pyrimethamine or cycloguanil was chosen to expose the synchronized stages of the parasites to determine the stage specific toxicity of the DHFR inhibitors to ring (Figure 5.2), trophozoite (Figure 5.3) and schizont (Figure 5.4) infected stages. Percent decrease in parasitemia observed at the end of the schizogonic cycle was maximum when late ring or trophozoite stages were under drug pressure (Figure 5.6). Rings were least susceptible to DHFR inhibitors. Marked difference in stage specific toxicity between pyrimethamine and cycloguanil was observed in group-5 where the parasites were exposed in schizont stage (36-48 hours) of the development. When the schizonts were exposed to these drugs 95% of subsequent ring stage production was inhibited by cycloguanil, whereas 56% inhibition was seen with pyrimethamine.

**Synergism**

The parasites of the highly pyrimethamine resistant line PS-5 derived from the clone F-56 were found to be more sensitive to pyrimethamine than to cycloguanil. The IC$_{90}$ values of the two drugs when evaluated alone were $8.6 \times 10^{-6}$ M for pyrimethamine and $3.2 \times 10^{-7}$ M for cycloguanil. Concentration of $4 \times 10^{-6}$ M of pyrimethamine and $2 \times 10^{-7}$ M of cycloguanil used in combination caused 90% inhibition of the parasite growth. Sum of the FIC values were close to 1 as calculated by the following formula of Berenbaum (1978):

$$\frac{4 \times 10^{-6}}{8.6 \times 10^{-6}} + \frac{2 \times 10^{-7}}{3.2 \times 10^{-7}} = 1.08$$

The sum of the FIC values for some other combinations of pyrimethamine and cycloguanil tested also gave figures close to 1. These results show no significant potentiation of antimalarial activity between cycloguanil and pyrimethamine against the asexual erythrocytic stages of *P. falciparum*. The combinations of the
two DHFR inhibitors, pyrimethamine and cycloguanil demonstrated at best only an additive effect.

**Biological advantage of pyrimethamine resistant parasites**

Table 5.5 shows the multiplication ability of pyrimethamine sensitive parent clone F-56 and the highly pyrimethamine resistant line PS-5 for two consecutive erythrocytic schizogonic cycles in vitro.

**Table 5.5** In vitro parasite multiplication as determined by multiplication factor (MF) and parasite multiplication rate (PMR) in multiwell tissue culture plates. Starting percentage parasitemia in F-56 and PS-5 were 0.82 and 0.68, respectively.

<table>
<thead>
<tr>
<th>Parasite</th>
<th>Erythrocytic schizogonic cycle</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>First</td>
<td>MF</td>
<td>PMR</td>
</tr>
<tr>
<td>F-56</td>
<td>5</td>
<td>0.75</td>
<td>2.86</td>
</tr>
<tr>
<td>PS-5</td>
<td>5.6</td>
<td>0.7</td>
<td>2.28</td>
</tr>
</tbody>
</table>

From the parasite multiplication rate (PMR) for the two consecutive schizogonic cycles it can be inferred that parasites of the sensitive clone and the resistant line have similar multiplication ability. Results of pyrimethamine sensitivity tests performed on mixed population of resistant and sensitive parasites at the beginning and at the end of 56 weeks of continuous cultivation in drug free medium, show no marked change in the susceptibility of the parasites to this drug.
DISCUSSION

Complete inhibition of multiplication of *P. falciparum* strains sensitive to chloroquine has been reported to take place in vitro at a concentration of 10 ng/ml (Nguyen Dinh and Trager 1980). Based on this observation both isolate FCD-4 and clone F-56 are chloroquine resistant, the clone being more sensitive than the parent isolate. Similarly the parasites capable of multiplying in a medium containing $10^{-7}$ M pyrimethamine (Smalley and Brown 1982) or cycloguanil (Watkins and Sixsmith 1984; Eriksson et al. 1989; Peterson et al. 1990) are likely to be resistant to this DHFR inhibitor to some extent. Amount of pyrimethamine or cycloguanil needed to achieve complete inhibition has been at least ten fold less than this concentration. Clearly, therefore, the parasites employed in this study are sensitive to the DHFR inhibitors. It has long been known that a single asexual parasite can give rise to both male and female gametocytes as well as more of asexual forms (Downs 1947). Some of the asexual parasites are incapable of giving rise to the sexual forms (Bhasin and Trager 1984). These mutants will not be able to propagate in nature beyond the host of their origin. Another novel phenotypic feature of the clone F-56 is that it does not form gametocytes in drug free medium, whereas the parent isolate FCD-4 is a good gametocyte forming line in vitro.

Pyrimethamine and cycloguanil are the two DHFR inhibitor antimalarial drugs developed, but pyrimethamine has been deployed on much larger scale than cycloguanil because of its longer retention time and smaller dose needed for cure (Bruce-Chwatt 1985). 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 (Bishop 1962; Diggens et al. 1970). The development of resistance to these inhibitors has been associated in the field with heavy use of monotherapy with the drugs in a particular area and emergence of resistance has been observed to be sporadic and multifocal. The ease of development of resistance to these drugs has
suggested that a mutation at a single locus is likely to be involved. Analysis of resistance to pyrimethamine and proguanil in experimental malaria, *P. gallinacium*, also suggests the involvement of a single gene (Bishop 1962). Resistance to pyrimethamine has been developed in *P. yoelii* in a single step by Diggens (1970) and later by Walliker et al. (1975) in *P. chabaudi*. Morgan (1974) obtained a pyrimethamine resistant line of *P. yoelii* from a clone of this species, demonstrating the origin of resistant mutants *de novo*. Cowman and Lew (1989) selected pyrimethamine resistant parasites of *P. chabaudi* in vivo by passaging parasites four times through mice treated with increasing doses of the drug. Banyal and Inselberg (1986) have been successful in obtaining pyrimethamine resistant lines of *P. falciparum* in vitro using two different protocols. In one of the protocols, they exposed the sensitive strain to increasing concentration of pyrimethamine in a series of selection steps while in the other, they first exposed the sensitive parasites in vitro to a mutagenic agent, the N-methyl-nitro-N-nitrosoguanidine (MNNG), and then to a single high dose of pyrimethamine for selection of resistant survivors. The relapse protocol adopted in the present study for selection of resistant lines to the two DHFR inhibitors is slow but very successful in selecting parasites exhibiting a very high level of resistance to pyrimethamine at least. The highest pyrimethamine resistant line obtained showed 2400 fold increase in IC\textsubscript{50} value compared to the parent clone. However, for reasons unknown we did not succeed in deriving parasite lines showing high level of resistance to cycloguanil. Failure to obtain cycloguanil resistance is quite intriguing in view of the fact that both pyrimethamine and cycloguanil are structurally somewhat similar, both are potent inhibitors of the DHFR enzyme of the *Plasmodium* and exert their toxic effect by binding to this enzyme. This observation is attractive from application point of view, as the experiments here have clearly demonstrated that in vitro emergence of pyrimethamine resistance is much faster and stronger than cycloguanil in *P. falciparum*. It should be of great interest to know, if under field condi-
tions also, the emergence of resistance to pyrimethamine is faster than cycloguanil in *P. falciparum*. No serious studies have been carried out in this regard, so far. It is not known whether cycloguanil and pyrimethamine share the same binding site on the DHFR enzyme, neither it is clear whether the acquisition of resistance to one of the drugs necessitates concomitant resistance to the other. The existence of field isolates resistant to both pyrimethamine and proguanil is well documented, so also the presence of natural isolates sensitive to one drug and resistant to the other and vice versa (Schapira 1984; Watkins et al. 1984). Recent reports on the molecular basis of pyrimethamine resistance in *P. falciparum* have implicated an accumulation of point mutations being associated with increasing resistance to pyrimethamine (Foote et al. 1990). Analysis of the *P. falciparum* DHFR from different parasites reveal the structural basis of differential susceptibility to these antifol drugs. Parasites harboring a pair of point mutations from Ala 16 to Val 16 and Ser 108 to Thr 108 are resistant to cycloguanil but not to pyrimethamine (Inselburg et al. 1988; Tanaka et al. 1990; Foote et al. 1990). A single Asn 108 mutation, on the other hand confers resistance to pyrimethamine with only a moderate decrease in susceptibility to cycloguanil (Cowman et al. 1988; Peterson et al. 1988; Snewin et al. 1989; Zolg et al. 1989). These and other related inferences are compiled from the studies made on parasites originating from different places. The molecular basis of resistance to cycloguanil and pyrimethamine of selected lines derived in the present study should provide more meaningful and accurate inferences to be drawn, as these lines have all originated from the same sensitive parent genome of the clone F-56.

An interesting observation made during the selection of antifol resistant parasite lines is that the non-gametocyte forming clone occasionally formed some gametocytes under drug pressure. A marked increase in the number of circulating gametocytes in *P. falciparum* infected patients has been reported following treatment with antimalarial drugs like pyrimethamine, proguanil and sulphadiazine
Increase in gametocyte production has also been observed in *P. gallinaceum* treated with sulphadiazine and proguanil, whereas no such increase has been recorded during treatment with chloroquine, mepacrine or pamquine (Bishop 1954). But these observations are based on in vivo systems and it is well known that the number of gametocytes produced during an infection is highly variable. The factors responsible for these variations have not been exactly identified. Therefore, there had always been suspicion regarding the increased number of gametocytes observed in vivo following treatment with DHFR inhibitors. This suspicion has been cleared by this study, as it has been amply demonstrated that DHFR inhibitors somehow induce the formation of gametocytes, even in a non-gametocyte forming clone.

No marked cross resistance to cycloguanil has been observed in parasites selected for low levels of resistance to pyrimethamine. But the highest pyrimethamine resistant line (PS-5) exhibits 1027 fold increase in cycloguanil IC$_{60}$ value. No cross resistance has been seen in the highest pyrimethamine resistant line (PS-5) to chloroquine or artemisinin. This is also expected as both chloroquine and artemisinin are structurally different from pyrimethamine and have dissimilar mode of actions. However, there is clear evidence of cross-resistance to methotrexate, another DHFR inhibitor. Concentration of $10^{-7}$ M of methotrexate causes complete inhibition of pyrimethamine sensitive clone F-56 in vitro whereas there is only 26% inhibition of pyrimethamine resistant line PS-5. Selection of methotrexate resistant *Leishmania* parasites in vitro has shown gene amplification leading to the overproduction of the DHFR enzyme in methotrexate resistant parasites (Coderre et al 1983), reminiscent of those observed in methotrexate resistant mammalian cells and fibroblasts (Haber et al 1981). Reduced binding of methotrexate to DHFR (5 to 10 fold) in parasite extracts has also been reported in pyrimethamine resistant *P. falciparum* parasites selected in vitro (Inselburg et
al. 1988). Part of the explanation for the decreased sensitivity of pyrimethamine resistant line to methotrexate may be attributed to the altered structure of the DHFR during the selection process that has reduced binding capability to methotrexate.

Selection of parasites exhibiting increased resistance to cycloguanil has been obtained relatively faster in the parasite from the parasites that have been selected earlier for pyrimethamine resistance than from the pyrimethamine sensitive parasites. This demonstrates that acquisition of resistance to pyrimethamine predisposes parasites to become resistant faster to a related antimalarial drug like cycloguanil, at least in vitro. Foote et al. (1990) postulate that factors responsible for pyrimethamine resistance may be genetically linked to cycloguanil resistance, since both these drugs show structural similarity and have similar mode of action. Cycloguanil resistance causing factors are perhaps not as actively selected as those of pyrimethamine and this might be responsible for less common occurrence of cycloguanil resistance.

A little studied aspect of drug resistance in malaria parasites is the capacity of the resistant forms to persist in the absence of drug pressure. This aspect is important especially in situations where the use of a particular drug has been discontinued for a long period with a hope that the resistant mutant forms would revert to sensitive phenotype in absence of drug pressure. This thinking is based on an assumption that on withdrawal of drug pressure resistant genes would be at a disadvantage or might not be of any use to the parasite and thus would be selected out in favour of some specific sensitive genes. However, contradictory observations have been made in this regard for instance, Jensen et al. (1981) have observed a case of clinical drug resistant falciparum malaria acquired from an initially chloroquine sensitive strain. A marked decrease in the level of sensitivity to chloroquine in falciparum has been observed after a few weeks of cultivation by Le Bras et al. (1983). Our studies with pyrimethamine resistant parasite lines
cultivated in drug free medium for an extended period of time in vitro, indicate no appreciable change in the level of sensitivity to pyrimethamine, clearly showing that resistance acquired by the parasites is stable and the resistant parasites do not show any biological advantage of overgrowing the sensitive parasites in vitro. Clyde (1967) has provided epidemiological evidence to the fact that drug resistance once arisen, can survive and spread in the absence of further drug treatment. It has been reported pyrimethamine resistant *P. falciparum* in regions of Tanzania several years after the large scale use of the drug has ended. Further there are evidences that the pyrimethamine resistance has spread from its presumed place of origin into surrounding areas in which no pyrimethamine is believed to have been used (Clyde 1967). Similar findings concerning cycloguanil are lacking.

The potentiating effects of pyrimethamine and sulfadiazine in treatment of *P. falciparum* infection in man has been demonstrated for the first time by Goodwin (1952). Drug combination of pyrimethamine and sulfadoxine (Fansidar) has been deployed exclusively for both therapy and prophylaxis in countries where multidrug resistant *P. falciparum* is a problem (Peters 1990c). A combination of pyrimethamine and dapson is also available under the name maloprim, though less popular (Worth and Werbel 1984). Unfortunately, resistance to these drug combinations have begun to emerge on a serious scale from various parts of the globe (Peters 1990b). In the present study it has been shown that there is no significant potentiating or synergistic antimalarial activity between cycloguanil and pyrimethamine when used in combination. Yeo and Rieckmann (1992) have also reported the absence of potentiation of antimalarial activity between cycloguanil and pyrimethamine. This, however, does not imply that the two drugs should not be used in combination. The identification of mutually exclusive point mutations in the DHFR gene encoding resistance to the two most widely used DHFR inhibitors (pyrimethamine and cycloguanil) and the observation that more extensive multiple mutations (that are
less likely to arise frequently) are required for resistance to both the drugs (Foote et al. 1990; Peterson et al. 1990), suggests an innovative approach to the deployment of a triple drug combination of pyrimethamine, cycloguanil, sulfadoxine to impede the spread of resistance in *P. falciparum* to the DHFR inhibitor antimalarials. The suggestion should be seriously considered for further in depth investigation.
Figure 5.1  The pattern of selection of progressively pyrimethamine-resistant (A) and cycloguanil-resistant (B) *P. falciparum* lines obtained in vitro from the clone F-56. The selective drug concentrations at which the parasites grew are shown. Beside the drug concentration, in parentheses is the number of days after the initial exposure to the drug that parasites grew normally in presence of drug concentration. The five pyrimethamine-selected lines, PS-1 to PS-5 growing at progressively higher concentrations are shown.

### A

Selection to pyrimethamine
F-56, initial IC$_{50}$ = 1.5 x 10$^{-9}$M

<table>
<thead>
<tr>
<th>Conc.</th>
<th>(days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10$^{-9}$M</td>
<td>(24)</td>
</tr>
<tr>
<td>5 x 10$^{-9}$M</td>
<td>(38)</td>
</tr>
<tr>
<td>10$^{-8}$M</td>
<td>(58)</td>
</tr>
<tr>
<td>5 x 10$^{-8}$M</td>
<td>(84)</td>
</tr>
<tr>
<td>10$^{-7}$M</td>
<td>(57)</td>
</tr>
<tr>
<td>5 x 10$^{-7}$M</td>
<td>(45)</td>
</tr>
<tr>
<td>10$^{-6}$M</td>
<td>(42)</td>
</tr>
</tbody>
</table>

### B

Selection to cycloguanil
F-56, initial IC$_{50}$ = 7.2 x 10$^{-11}$M

<table>
<thead>
<tr>
<th>Conc.</th>
<th>(days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 x 10$^{-11}$M</td>
<td>(32)</td>
</tr>
<tr>
<td>8 x 10$^{-11}$M</td>
<td>(29)</td>
</tr>
<tr>
<td>10$^{-10}$M</td>
<td>(20)</td>
</tr>
<tr>
<td>2 x 10$^{-10}$M</td>
<td>(47)</td>
</tr>
<tr>
<td>4 x 10$^{-10}$M</td>
<td>(58)</td>
</tr>
<tr>
<td>6 x 10$^{-10}$M</td>
<td>(54)</td>
</tr>
<tr>
<td>10$^{-9}$M</td>
<td>(67)</td>
</tr>
<tr>
<td>2 x 10$^{-9}$M</td>
<td>(44)</td>
</tr>
</tbody>
</table>
Figure 5.2  A Geimsa stained thin blood film from *P. falciparum* synchronised culture showing ring stages (0-7 hours old).
Figure 5.3  A Geimsa stained thin blood film from *P. falciparum* synchronised culture showing trophozoite stages.
Figure 5.4 A Geimsa stained thin blood film from *P. falciparum* synchronised culture showing schizont stages.
Figure 5.5 Spontaneously released merozoites from a ruptured schizont in culture surrounding the free pigment.
Figure 5.6 Sensitivity to pyrimethamine and cycloguanil in vitro of different synchronised *P. falciparum* stages, rings (0-12 hours), early trophozoites (12-24 hours), trophozoites (24-36 hours) and schizonts (36-48 hours).
Abstract: Feasibility of assay methods for diagnosis of Plasmodium falciparum resistance to DHFR inhibitor antimalarials 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. 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.

INTRODUCTION

Point mutations, primarily within the gene sequence coding for the dihydrofolate reductase of P. falciparum, have been shown to impart pyrimethamine resistance to these parasites (Peterson et al. 1988; Cowman et al. 1988; Zolg et al. 1989; Tanaka et al. 1990). The resistant parasites produce altered DHFR enzyme which retains its biocatalytic activity but loses the characteristic affinity binding of pyrimethamine (McCutchan et al. 1984; Walter 1986). Sequence comparison of DHFR gene from pyrimethamine sensitive and resistant falciparum lines indicate the pivotal role of amino acid 108 in the resistance mechanism to this antifol. A single base exchange in position 323 of the DHFR coding region changes Thr-108 or Ser-108 found in pyrimethamine sensitive parasites to Asn-108. Additional point mutations in the DHFR gene have been found to confer higher degree of resistance (Foote et al. 1990). Due to their subtle nature, point mutations are the most difficult to detect of all the genetic alterations by methods other than
sequencing. The development of technologies for detection and characterization of these genetic alterations is having great impact in early cancer diagnosis, since some point mutations have been implicated in several malignancies (Carter et al. 1992; van Mansfeld and Bos 1992; Tominaga et al. 1992). The other area where the potential of these technologies hold promise is the early detection of incipient drug resistance, arising out of point mutations due to drug pressure, in parasite populations and in understanding the mechanisms of drug resistance. Methods currently utilized for detection and characterization of single base substitution, deletion or insertion, other than sequencing are: a) RNAase mismatch cleavage methods (Winter et al. 1985; Myers et al. 1985). b) The mismatch chemical cleavage (MCC) by generic denomination (Cotton et al. 1988; Montandon et al. 1989; Gogos et al. 1990). c) The single strand conformation polymorphism (SSCP) (Hayashi 1991). d) Restriction fragment length polymorphisms (RFLP). e) Mutation specific PCR. With the exception of the MCC, all these methods have been successfully used with total genomic or total cellular RNA. They have been greatly simplified by use of previously amplified nucleic acids in vitro by polymerase chain reaction (PCR). In vitro test methods have widely been used for determining the drug sensitivity of *P. falciparum* isolates (Nguyen-Dinh and Payne 1980; Richards and Maples 1979; Yisunsri and Rieckmann 1980) but there are several limitations that affect their usefulness (Desjardins 1984). Feasibility of alternate assay methods based on RFLP and PCR are explored. This chapter will address the application of the RFLP to the detection of genomic rearrangements in drug resistant parasites and utility of mutation specific PCR to distinguish the pyrimethamine sensitive and resistant parasite lines selected in vitro.

**MATERIALS AND METHODS**

**Parasite cultures** Parasites of the clone F56 and the five pyrimethamine resistant lines (PS-1 to PS-5) derived from it were each seperately grown in large
glass Petridishes (10 cm diameter) using candle jar method (Trager and Jensen 1976). When parasitemia in these cultures reached 5-6%, the cells were pelleted, washed once with HEPES buffered RPMI-1640 medium, snap frozen in liquid nitrogen and stored below 0 °C till DNA was extracted. Before freezing, a cell count was made in a haemocytometer and total parasite count was estimated in each sample.

**Requirements for extraction of parasite DNA**

**RNAase A (10mg/ml):** Pancreatic RNAase was dissolved at a concentration of 10 mg/ml in 10 mM Tris.Cl (pH7.5), 15mM NaCl. Solution was heated to 100 °C for 15 min, allowed to cool slowly at room temperature, dispensed in aliquots and stored at -20 °C.

**DNA extraction buffer:** It consisted of the following reagents-
- 10mM Tris.Cl (pH 8.0)
- 0.1M EDTA (pH 8.0)
- 20 μg/ml Pancreatic RNAase A
- 0.5% SDS

**Proteinase K (20mg/ml):** Stock solution of proteinase K was prepared at a concentration of 20 mg/ml in water and stored at -20 °C.

**Phenol:** Liquified phenol stored at -20 °C was melted at 68 °C, mixed with hydroxyquinoline (final concentration 0.1% ) and equilibrated with 0.1M Tris.Cl (pH 8.0). It was stored in a dark bottle under a layer of 0.1M Tris.Cl (pH 8.0) containing 0.2% β-mercaptoethanol at 4 °C.

**Chloroform:** It was used in combination with isoamylalcohol in the ratio of 24:1.

**TE buffer (pH 7.5):** It had the following composition
- 10mM Tris.Cl (pH 7.5)
- 1mM EDTA (pH 8.0)

The buffer was sterilized by autoclaving and stored at room temperature.

**TBE buffer (5X):** One litre of 5X TBE was prepared by mixing 54 g Tris base,
27.5 g boric acid and 20 ml of 0.5 M EDTA (pH 8.0) in water.

**Extraction of parasite DNA:** The frozen samples (containing about $10^{10}$ parasites) were thawed, mixed with two volumes of 1% cold acetic acid and centrifuged at 6000 rpm for 20 min at 4 °C. Supernatant which contained most of the haemoglobin was removed and the pellet was resuspended in two volumes of 1% triton X-100. It was again spun at 6000 rpm for 20 min at 4 °C and supernatant discarded. The resulting pellet of parasites was washed twice with 0.85% NaCl (pH 7.4). The washed parasite pellet was again resuspended in DNA extraction buffer in a volume equal to that of the original frozen sample and incubated at 37 °C for 1 hour with intermittent shaking. Proteinase K at a final concentration of 100 μg/ml was added to the above mixture, mixed gently and incubated overnight at 37 °C. DNA was extracted with an equal volume of phenol once, phenol:chloroform (1:1) twice, chloroform:isoamylalcohol (24:1) once and chloroform once. To the aqueous phase of the final extraction step was added 0.2 volume of 10 mM ammonium acetate and 2.5 volume of cold absolute alcohol (ethanol). This was left overnight at -20 °C for DNA to precipitate. DNA was pelleted by centrifugation at 12,000 rpm for 30 min at 4 °C and supernatant carefully removed. The pellet was washed with cold 70% ethanol and dissolved in 200 μl of TE buffer (pH 7.5). Amount of DNA was estimated spectrophotometrically at optical density 260 nm and its purity determined from ratio of $OD_{260}/OD_{280}$. The presence of contaminating RNA and sheared DNA, if any, was checked by running the samples on a mini agarose gel.

**Components of Southern hybridization and oligonucleotide probe labelling**

The stock solutions and buffers were prepared in glass redistilled water according to the protocols of Sambrook et al. (1989)

**SSC(20X):** Sodium chloride and sodium citrate were dissolved in water to get final concentration of 3M and 0.3M, respectively. The pH of the solution was adjusted to 7.0 with NaOH.
**STE buffer** (pH 7.5): It consisted of the following reagents

100 mM NaCl
10 mM Tris.Cl (pH 7.5)
1 mM EDTA (pH 8.0)

The buffer was sterilized by autoclaving and stored at room temperature.

**Sephadex G-50**: Sephadex G-50 slurry was made by mixing 3 g of it with 300 ml of STE solution and the suspension was autoclaved to hydrate the beads.

**Denhardt’s solution** (100X): 2 g each of Ficoll, polyvinylpyrrolidone (PVP-40, Sigma) and BSA was dissolved in 100 ml of distilled water and stored at -20°C.

**Sodium phosphate buffer** (1 M, pH 7.2): It was prepared by mixing 28 ml of 1M NaH₂PO₄ solution and 72 ml of 1M Na₂HPO₄ solution.

**Pre-hybridization buffer**: The composition of the buffer was as follows

5 X SSC
5% (w/v) Dextran sulfate
0.05M Sodium phosphate buffer (pH 7.2)
5X Denhardt's solution
0.0025M EDTA
0.4% SDS

It was stored in aliquots at -20°C.

**Salmon sperm DNA** (5 mg/ml): It was dissolved at a concentration of 5 mg/ml in water and stirred continuously to get complete dissolution. DNA was sheared by repeatedly passing it through a syringe with 18 gauge needle and stored in aliquots at -20°C.

**Loading dye** (6X): It had the following composition

0.25% Bromophenol blue
0.25% Xylene cyanol FF
40% (w/v) Sucrose in water

**Restriction enzymes and their buffers**: The following 10 X restriction en-
zyme buffers were prepared for use with the respective restriction enzymes

<table>
<thead>
<tr>
<th>Restriction enzyme</th>
<th>Composition of 10 X buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Alu I</em></td>
<td>10 mM Tris.Cl (pH 7.0)</td>
</tr>
<tr>
<td></td>
<td>10 mM MgCl₂</td>
</tr>
<tr>
<td></td>
<td>1 mM DTT</td>
</tr>
<tr>
<td><em>Hind III</em></td>
<td>50 mM NaCl</td>
</tr>
<tr>
<td></td>
<td>10 mM Tris.Cl (pH 7.9)</td>
</tr>
<tr>
<td></td>
<td>10 mM MgCl₂</td>
</tr>
<tr>
<td></td>
<td>1 mM DTT</td>
</tr>
<tr>
<td><em>Taq I</em></td>
<td>100 mM NaCl</td>
</tr>
<tr>
<td></td>
<td>10 mM Tris.Cl (pH 8.4)</td>
</tr>
<tr>
<td></td>
<td>10 mM MgCl₂</td>
</tr>
<tr>
<td></td>
<td>10 mM β-mercaptoethanol</td>
</tr>
<tr>
<td></td>
<td>BSA was added at a final</td>
</tr>
<tr>
<td></td>
<td>concentration of 100 μg/ml in 1X reaction mixture</td>
</tr>
</tbody>
</table>

**Bacteriophage T₄ polynucleotide kinase (PNK) buffer:** PNK, 10X buffer was made of the following composition

- 500 mM Tris.Cl (pH 7.5)
- 100 mM MgCl₂
- 50 mM DTT
- 1 mM EDTA (pH 8.0)
- 1 mM Spermidine HCl

**RFLP using Southern hybridization**

*Restriction digestion of DNA:* DNA about 4 μg each from the clone F-56 and the pyrimethamine resistant lines was individually digested with 20 units of either *Hind III*, *Alu I* or *Taq I* restriction endonuclease in an appropriate buffer in a total
volume of 20 μl. Restriction digestion reaction was carried for three hours. Reaction mix containing Taq I was incubated at 65 °C while others at 37 °C. Following incubation the microfuge tubes containing Hind III or Alu I were transferred to a 65 °C water bath for 10 min and kept in ice for 5 min.

*Preparation of Southern blots:* The entire volume of each restriction digestion (20 μl) was mixed with 4 μl of 6 X loading dye, loaded on a 0.8% agarose gel made in 0.5 X TBE buffer and electrophoresed in the same buffer overnight at 40 volts till the tracking bromophenol dye had travelled 20 cm from the well. Gel was stained in 1 μg/ml ethidium bromide solution for 30 min, visualised under UV light and photographed with a polaroid camera. DNA was transferred to a nylon membrane by Southern's capillary method as follows:

Size fractionated DNA was depurinated by incubating the gel in 0.25 N HCl solution at room temperature with gentle agitation for 15 min. Acid solution was decanted and replaced with 0.4 N NaOH solution and gel incubated with constant agitation for 30 min. Gel was then neutralised in 12 mM Tris, 6 mM sodium acetate, 0.3 mM EDTA solution (pH 7.5) for 30 min with gentle shaking of the gel. Gene screen membrane cut to the size of gel was wetted with distilled water by capillary action and immersed in transfer buffer (10 X SSC) for 15 min. A piece of Whatman 3MM paper was wrapped around a stack of three glass plates and placed inside a large baking dish. The dish was filled with transfer buffer (10 X SSC) until the level of liquid reached almost to the top of glass plates. Gel was removed from the neutralising solution, inverted and placed on the glass support kept in baking dish. All the edges of the gel were covered with a thin strip of parafilm to prevent liquid from flowing directly from reservoir to paper towels placed on top of the gel. Wet nylon membrane was then placed on the gel. Two pieces of Whatman 3MM paper cut to the size of gel were dipped in 2 X SSC and placed on top of the membrane. Care was taken that no air bubble was trapped between any of the layers in the entire set-up. A stack of paper towels (5 cm high), just smaller than
the size of Whatman paper was placed on top of Whatman paper. Placed a glass plate on top of the stack and weighed it down with a 500 g weight. The transfer of DNA was allowed to proceed for 20-24 hours with periodic replacement of wet paper towels with dried ones. Membrane was carefully removed after the transfer and soaked in 6 X SSC for 5 min before being dried at room temperature. Gel was restained in ethidium bromide solution (1µg/ml) and viewed under UV lamp to check the efficiency of transfer. Air dried membrane was sandwiched between two sheets of dry 3MM Whatman paper and baked in vacuum oven at 80 °C for 2 hours.

End labelling of synthetic oligonucleotide employed as a probe
The synthetic 21-mer deoxynucleotide which was obtained as lyophilized powder had the following sequence:

5'- AGGTCTTAACTTGACTAACAT -3'

It was dissolved in 500 µl of sterile distilled water, vortexed and centrifuged to remove the remaining resin. The aqueous phase containing dissolved oligonucleotide was extracted once with phenol:chloroform (1:1) and once with chloroform. 0.1 volume of 0.1M MgCl₂ and 2 volumes of ethanol were added to aqueous phase of chloroform extraction and left overnight in -20 °C freezer for oligo- precipitation. Oligomer was recovered by centrifuging at 14,000 rpm for 30 min at 4 °C in a microfuge. The pellet was washed twice with 70% ethanol and finally dissolved in 200 µl of sterile distilled water and quantitated spectrophotometrically. About 15 pM (0.1µg) of oligonucleotide was labelled at 5' end with 10 µCi of (γ-³²P) ATP (specific activity 3000 Ci/mmol) using 20 units of T₄ polynucleotide kinase (PNK) in PNK buffer in a reaction volume of 50 µl at 37 °C for 1 hour. The reaction was terminated by adding 2 µl of 0.5 M EDTA.

Separation of labelled oligo probe from unincorporated nucleotides
The oligo probe was separated from unincorporated nucleotides using Sephadex G-50 spun columns. The spun column was prepared as follows. Bottom of a 1ml
disposable syringe was plugged with autoclaved glass wool. Sephadex G-50 slurry, equilibrated in STE was inserted into a centrifuge tube, containing at the bottom an eppendorf tube with its lid cut off and this set-up was centrifuged at 3,000 rpm for 5 min. Eluate from centrifuge tube was drained out, more of slurry was added into syringe and this was repeated 2-3 times till the post-spin bed volume of 0.9 ml was obtained. 100 μl of STE was then added into syringe and spun at the same speed (3000 rpm) for the same time. This was repeated once more. The eppendorf tube in centrifuge was replaced with a fresh one. The column, when not needed immediately, was stored at 4 °C after wrapping the mouth of syringe with parafilm. End labelled probe was made to 100 μl volume by adding STE solution. 100 μl of labelled probe was loaded on to each of the spun column and centrifuged at 3000 rpm for 5 min. The eluate was collected in an eppendorf tube and syringe was safely disposed off in a radioactive waste container. 1μl of this purified probe was taken in an eppendorf tube and placed in a scintillation vial for Cerenkov counting in a scintillation counter (Beckman Instruments Inc.). The specific activity of probe was calculated by following formula.

The specific activity of probe = (CPM for 1μl sample) x (counting efficiency factor) x (volume of end labelled probe in μl) x 10

When not used immediately, the labelled probe was stored in a lead container in -20 °C freezer and was used within 4-5 days.

Hybridization on the Southern blot

Prehybridization: Each of the Southern blotted nylon membrane was individually placed in heat sealable plastic bag. Prehybridization buffer (80 μl buffer/ cm² area of membrane) was added into the bag. Salmon sperm DNA was denatured by boiling for 10 min and added into the bag (50 μg salmon sperm DNA/ml prehybridization buffer). It was thoroughly mixed and the bag was sealed after removing all air bubbles. Membrane was prehybridized by keeping the bag in a waterbath set at 65 °C for 3-5 hours.
**Hybridization:** Labelled oligomer probe was boiled for 10 min and quickly cooled on ice for 10 min. One corner of the bag was cut open and probe was added. Bag was resealed after removing air bubbles and hybridized overnight at 40 °C in a waterbath with constant and slow shaking.

**Post-hybridization washing of membrane:** Membrane was removed from the bag and incubated in a solution of 2X SSC, 0.1% SDS at room temperature for 8 min with constant shaking and this step was repeated once more. The above solution was replaced with prewarmed (40 °C) solution of 0.5X SSC, 0.1% SDS and incubated in a shaker water bath at 40 °C for 15 min. This was repeated with fresh prewarmed solution of 0.5X SSC, 0.1% SDS. Membrane was then washed twice with 2X SSC at room temperature with constant shaking for 8 min each.

After the final wash, membrane was placed on a sheet of prewet (with distilled water) Whatman 3 mm paper, covered with cling film (Saran wrap) and exposed to X-ray film (Kodak XOMAT AR) for desired duration of time, using intensifying screens at -70 °C. X-ray film was developed by immersing it in developer (20% Kodak GBX) for 1 min, rinsed briefly in water, immersed in fixer (20% Kodak GBX) for 3 min and finally rinsed in running tap water for 5 min. X-ray film was dried and photographed.

**Polymerase chain reaction** (Mullis 1986; Saiki et al. 1988)

**The technique:** This is a technique used for specific in vitro amplification of nucleic acid segment that is bracketed between two regions of known sequences. The hallmarks of this method include specificity, sensitivity and speed beside being straightforward. The specificity of the amplification results from the requirement of DNA polymerases for a primer (a short stretch of synthetic nucleotides) that is extended only when annealed to a complementary target sequence. In presence of dNTPs, the annealed primers are extended from 5' to 3' end and the target DNA strand is copied from 3' end by the DNA polymerase during this extension step. The copying is halted by raising the temperature to 94 °C, causing
separation of all double stranded molecules. This denaturation step is followed by
annealing step in a typical cycle of the polymerase chain reaction wherein the
reactants are cooled to 37 - 55 °C, the temperature which favours the annealing of
the primers to template DNA. Raising the temperature again to 72 °C stimulates
the heat resistant Taq polymerase to copy the target DNA by extending the an­
nealed primers. Number of copies of the target DNA increases in an exponential
fashion with each subsequent cycle. Usually 30 cycles are carried out giving an
amplification level of $10^6$.

Components of the polymerase chain reaction:

10X reaction buffer

The reaction buffer had the following composition:

100 mM Tris-HCl pH 8.3
500 mM KCl
15 mM MgCl₂
0.01% (w/v) gelatin (Sigma, Cat. No. G2500, St. Louis, MO)

Deoxynucleotide triphosphates (mix)

Stock solution of each of the following dNTPs was prepared in water (pH 7.0)

- dATP 10 mM
- dCTP 10 mM
- dGTP 10 mM
- dTTP 10 mM

Each of the dNTP was included at a final concentration of 200 μM in the reaction
mix.

Template DNA

Dilutions of the template DNA from the stock were made in 10 nM Tris.Cl pH 8.0,
1mM EDTA pH 8.0 solution. About 70 ng of template DNA was included in each
reaction mix.

Primers employed for the polymerase chain reaction
1. Control primers

Bacteriophage lambda DNA was used as control DNA template. Oligonucleotides used as primers in the amplification of a segment of λ DNA were

<table>
<thead>
<tr>
<th>Primer</th>
<th>Map position</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control primer 1</td>
<td>7131-7135</td>
<td>5'-GATGAGTTCTGGATCAGACTG-3'</td>
</tr>
<tr>
<td>Control primer 2</td>
<td>7606-7630</td>
<td>5'-GGTTATCGAAATCAGCCACAGCGGTCAGC-3'</td>
</tr>
</tbody>
</table>

From map positions, these two primers should amplify 500 nucleotide target segment of the λ DNA.

2. Mutation specific primers

Mutation specific primers employed to discriminate pyrimethamine sensitive and resistant *P. falciparum* lines by polymerase chain reaction were:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Type</th>
<th>Map position</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP</td>
<td>Detects the Ser 108 codon</td>
<td>323-337</td>
<td>5'-GAATGCTTTCCCAGC-3'</td>
</tr>
<tr>
<td></td>
<td>of pyrimethamine sensitive parasites</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DR1</td>
<td>Detects the Asn-108 codon</td>
<td>323-337</td>
<td>5'-GAATGCTTTCCCAGG-3'</td>
</tr>
<tr>
<td></td>
<td>of pyrimethamine resistant parasites</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP</td>
<td>Counterprimer used with SP and DR1</td>
<td>1-21</td>
<td>5'-ATGATTGGAAAGCTTGAAGCGAC-3'</td>
</tr>
</tbody>
</table>

These oligonucleotide primers were synthesized at the Keystone laboratories and were derived from the DHFR coding sequence of the *P. falciparum* genome (Bzik et al. 1987). Additional primers evaluated for amplification with the above counterprimer (CP) were:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Type</th>
<th>Map position</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>DR2</td>
<td>Detects the Thr-108 codon</td>
<td>323-337</td>
<td>5'-GAATGCTTTCCCAGG-3'</td>
</tr>
<tr>
<td></td>
<td>of cycloguanil resistant parasites</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
DP A distant primer present both in sensitive and resistant lines

1154-1169 5'-CACCTACTCCCGTTCG-3'

Taq DNA polymerase

The enzyme was used at a concentration of 2.5 units/100μl reaction mix.

Equipments

1. Thermal cycler, Perkin Elmer Cetus DNA Thermal Cycler Model 480.
2. Electrophoresis apparatus to characterize amplification products on agarose gels.
3. Transilluminator with polaroid camera

Mutation specific polymerase chain reaction  The components of the amplification reactions were mixed in the following order in a sterile 0.5 ml microfuge tube:

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autoclaved redistilled water</td>
<td>30 μl</td>
<td></td>
</tr>
<tr>
<td>10X Reaction buffer</td>
<td>10 μl</td>
<td>1X</td>
</tr>
<tr>
<td>dATP</td>
<td>2 μl</td>
<td>200μM</td>
</tr>
<tr>
<td>dCTP</td>
<td>2 μl</td>
<td>200 μM</td>
</tr>
<tr>
<td>dGTP</td>
<td>2 μl</td>
<td>200 μM</td>
</tr>
<tr>
<td>dTTP</td>
<td>2 μl</td>
<td>200 μM</td>
</tr>
<tr>
<td>Primer 1(CP)</td>
<td>1 μl</td>
<td>500 ng</td>
</tr>
<tr>
<td>Primer 2 (SP,DR1,DR2 or DP)</td>
<td>1 μl</td>
<td>500 ng</td>
</tr>
<tr>
<td>Template DNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Made the final volume to 100 μl with water</td>
<td>70 ng</td>
<td></td>
</tr>
</tbody>
</table>

Depending on the conc. of the stock
Positive control for PCR  Whole bacteriophage lambda DNA was used as a positive control template DNA. The positive control reaction mix consisted of the following components:

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autoclaved redistilled water</td>
<td>62 µl</td>
<td></td>
</tr>
<tr>
<td>10X Reaction buffer</td>
<td>10 µl</td>
<td>1X</td>
</tr>
<tr>
<td>dATP</td>
<td>2 µl</td>
<td>200 µM</td>
</tr>
<tr>
<td>dCTP</td>
<td>2 µl</td>
<td>200 µM</td>
</tr>
<tr>
<td>dGTP</td>
<td>2 µl</td>
<td>200 µM</td>
</tr>
<tr>
<td>dTTP</td>
<td>2 µl</td>
<td>200 µM</td>
</tr>
<tr>
<td>Control primer 1</td>
<td>5 µl</td>
<td>1.0 µM</td>
</tr>
<tr>
<td>Control primer 2</td>
<td>5 µl</td>
<td>1.0 µM</td>
</tr>
<tr>
<td>Control template</td>
<td>10 µl</td>
<td>1ng</td>
</tr>
<tr>
<td>(1:10 dilution)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total mix</td>
<td>100 µl</td>
<td></td>
</tr>
</tbody>
</table>

Overlaid the reaction mixture with 50 µl of light mineral oil to prevent evaporation of the sample during repeated cycles of heating and cooling. The reaction mixture was heated for 5 min at 94 °C to denature the DNA completely. While the reaction was still at 94 °C, 0.5 µl (5 units/µl; Perkin Elmer Cetus N801-0046) of Taq DNA polymerase was added. The amplification reaction was carried out as described below. Typical conditions for denaturation, annealing and polymerization were standardized and used as follows:

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Denaturation</th>
<th>Annealing</th>
<th>Polymerization</th>
</tr>
</thead>
<tbody>
<tr>
<td>First cycle</td>
<td>5 min at 94 °C</td>
<td>45 sec at 58 °C</td>
<td>2 min at 72 °C</td>
</tr>
<tr>
<td>Subsequent cycles</td>
<td>45 sec at 94 °C</td>
<td>45 sec at 58 °C</td>
<td>1 min at 72 °C</td>
</tr>
<tr>
<td>Last cycle</td>
<td>45 sec at 94 °C</td>
<td>45 sec at 58 °C</td>
<td>8 min at 72 °C</td>
</tr>
</tbody>
</table>

The duration of the ramp temperature was 30, 30 and 45 seconds between
each denaturation, annealing and polymerization step of a cycle, respectively. Amplification was carried for a total of 28 cycles.

**Analysis of PCR products:** Following amplification the amplified product from the reaction mixture was examined by gel electrophoresis. 10 µl sample of the amplified DNA was drawn from each of the microfuge tube and mixed with 2 µl of 6X loading dye. The samples were then loaded on an agarose gel (1.5 %) made in 0.5X TBE buffer containing ethidium bromide (0.5 µg/ml) and electrophoresed in the same buffer at 60 volts till the tracking bromophenol dye travelled a distance of atleast 40 to 45 mm from the well. The gel was visualized in dark under UV illumination and photographed.

**RESULTS**

**Extraction of falciparum DNA**

DNA extracted from the parasites was found to be of high molecular weight and examination of various samples on the agarose gel stained with ethidium bromide revealed that there was no visible shearing of the DNA samples isolated. Quality of the DNA samples obtained was further assessed by determining the OD ratio of each sample at 260 and 280 nm. The OD_{260}/OD_{280} was found to be close to 1.8 in each case. This purified DNA was employed for all further work.

**Restriction digestion of DNA**

Four microgram DNA each from the clone F-56 and the pyrimethamine selected lines were individually digested with either Hind III, Alu I or Taq I restriction enzyme. The digested material was size fractionated on a 0.8% agarose gel. The gels were stained with ethidium bromide and photographed. Lane 1 of Figure 6.1 shows the uncut F-56 DNA as clear single band without smear or contaminating RNA. The F-56 DNA digested with Hind III, Alu I and Taq I for three hours were loaded in lane 2,3 and 5, respectively. The lane 4 shows the DNA digested for one hour with Taq I and Hind III cut lambda DNA showing discrete bands is seen
in lane 6. Presence of smears in lanes 2 to 5 shows that the DNA used was susceptible to the digestion by all the enzymes used. Digestion was more extensive in three hours of incubation. Alu I generated smaller fragments than the other enzymes.

**RFLP analysis** Autoradiograms showing specific hybridization of blot transferred DNA molecules onto nylon membrane to the radiolabelled oligomer probe is seen in the form of prominent dark bands or smears (Figures 6.2 to 6.4). Alu I digested DNA from pyrimethamine selected lines PS-5, PS-4, PS-3, PS-2 and sensitive clone F-56 were loaded in the gel lanes 1, 2, 3, 4 and 5, respectively of the autoradiogram (Figure 6.2). Comparison of the band profile of the most resistant line (PS-5) with the sensitive parent clone F-56 shows the presence of additional bands in resistant line. Arrangement of DNA bands looks almost alike in Figure 6.3 of the Hind III digested DNA of resistant and sensitive cell lines. Considerable difference in the hybridization patterns can be seen in Taq I digested DNA obtained from sensitive and resistant lines probed with the repeat oligomer. Several new bands made their appearances in the most resistant cell line which were conspicuous by their absence in the genome of sensitive F-56 clone and other selected lines.

**Mutation specific polymerase chain reaction**

Mutation specific PCR is the variation of the basic PCR theme. It is a method for determining whether previously characterized point mutations are present in a target sequence. The assay is based on the observation that efficient amplification under stringent PCR conditions occurs only when there is a perfect match between the target DNA and the 3' terminus of the diagnostic primer. A single nucleotide change can thus be detected by a PCR primer having 3' terminal nucleotide complementary to the mutation. As shown below the fully complementary primer I will be extended by Taq polymerase while primer II mismatched at the 3' terminus will not be extended:
ATG CAT ATT GCG TCG TCC ———> Matched primer I
TAC GTA TAA CGC AGC AGG GCA TTG ACG CTA ATT GGA CTG A (target DNA)

ATG CAT ATT GCG TCG TCC ———» X ———> Mismatched primer II
TAC GTA TAA CGC AGC AGT GCA TTG ACG CTA ATT GGA CTG A (target DNA)

Two diagnostic primers and one common primer are needed for the detection of each point mutation; one diagnostic primer complementary at the 3' terminal nucleotide to the wild-type sequence, the other complementary to the mutated sequence.

Design of mutation-specific primers

The mutation-specific primer assayed to detect the presence of Ser 108 (codon AGC), located in the DHFR-TS gene of the pyrimethamine sensitive parasites, was a 15-mer oligonucleotide and ended with cytosine base at the 3' annealing position 323, which is the central nucleotide of the 108 serine codon AGC. The SP primer 5'-GAATGCTTTCCCAGC-3' is fully complementary to the wild type pyrimethamine sensitive DHFR gene segment. The primer used to locate asparagine (Asn) codon AAC of resistant parasites was similar to the SP primer except that it had thymine base terminus at 3' end (DR-1 primer). Another drug resistant diagnostic primer DR-2 had guanine base at 3' terminus and amplifies the target DNA if threonine codon (ACC) was present, found in the cycloguanil resistant parasites at 323 position of the DHFR gene. All these mutation specific primers when used in combination with a 21-mer oligonucleotide counter primer (CP) annealing with the starting portion of DHFR coding sequence results in amplification of 337 base pair fragment by PCR. A distant 16-mer primer (DP) spanning between 1154-1169 nucleotides of the DHFR coding region present both in
sensitive and pyrimethamine resistant parasites had also been used in combination with the CP for amplification.

Detection limit of a 15-mer primer

Taking variable amounts of target DNA from F-56, ranging between 0.001 ng to 100 ng with a 10 fold difference, an experiment was performed to estimate the minimum amount of the DNA required for amplification with a pair of primers, the SP a 15-mer and the CP a 21-mer, so as to have a visible fluorescence on an agarose gel on loading 10 μl aliquot of the total 50 μl of the amplified product obtained after 30 cycles of PCR. A typical time and temperature profile of the cycle was 45 sec at 94 °C, 45 sec at 58 °C and 1 min at 72 °C. As seen in the Figure 6.5 even 1 pg of the target DNA was sufficient to give a clear fluorescent band on loading only 20 % of the amplified product.

Amplification of λ control DNA template

Amplification conditions employed efficiently amplified a specific 500 base pair target segment bracketed between a pair of control primers 1 and 2 from the 48.5 kb λ genome as seen in lane 3 of Figure 6.8, for instance.

Detection of pyrimethamine resistance with mutation specific primers

A series of experiments were performed to determine the most stringent annealing temperature that can be used at which the mutation specific primers were able to prime the enzymatic amplification and at the same time discriminate the point mutation at position 323 of the DHFR coding region of the pyrimethamine resistant and sensitive parasites. The annealing temperature was increased by 1 °C in each experiment, beginning from initial annealing temperature of 45 °C. Effective amplification and discrimination was obtained at an annealing temperature of 58 °C. Using the primer pair CP and SP, which form perfect match for wild type Ser codon (AGC) at the position 323 of the DHFR coding region, efficient amplification was found in the microfuge tubes containing target DNA F-56, PS-1 and PS-2 amplification product shown in lanes 4, 5 and 6, respectively of Figure 6.6. No
amplification whatsoever was seen in lanes 6, 7 and 8 which contained the target DNA derived from PS-3, PS-4 and PS-5 pyrimethamine selected lines. This pair of primers can clearly, therefore, discriminate between sensitive F-56, PS-1 and PS-2 lines from the resistant PS-3, PS-4 and PS-5 lines without any ambiguity. Similarly by employing CP and DR-1 pair of primer in each tube containing target DNA from either F-56, PS-1, PS-2, PS-3, PS-4 or PS-5 lines of *P. falciparum*, enormous amplification of target DNA obtained from PS-3, PS-4 and PS-5 pyrimethamine resistant lines as can be observed in lanes 6, 7, 8 of Figure 6.7, respectively. This pair of primer can not amplify the DNA from sensitive lines (F-56, PS-1, PS-2) but is very efficient in amplifying DNA from pyrimethamine resistant lines (PS-3, PS-4, PS-5). The expected specific amplification of the target DNA was monitored by the molecular weight of the amplified product by running parallel controls. The two pairs of primers (one common and the two diagnostic) used had been found to be very effective in discriminating the target DNA obtained from pyrimethamine sensitive and in vitro selected resistant cell lines. In another set of experiment Figure 6.8 using different sets of primers (even multiplex), target DNA etc. the utility of CP and SP pair was reconfirmed, in amplifying only the pyrimethamine sensitive target DNA only whereas all the other primer and target DNA combinations failed to show the amplified product. The primer pair CP and DR-2 failed to amplify the target DNA obtained from F-56 or any of the selected parasite lines.

**DISCUSSION**

Genetic characterization of microorganisms is being performed increasingly at the DNA level. The molecular biology approach generally used for an isolate or clone individualization which may be phenotypically alike is the typing of variable number of tandem repeat loci by restriction fragment length polymorphism. How far this and other methods are valid and practical to detect drug resistance
resulting from point mutations in *P. falciparum*? The methods currently in use to detect and characterize single base change in a gene are: a) Mismatch cleavage by RNAase. This method is based on the ability of pancreatic ribonuclease to recognize and cleave single base mismatches in RNA:RNA (Winter et al. 1985) and RNA:DNA heteroduplexes (Myers et al. 1985). The method is performed by hybridization of the target sequence to a labelled complementary riboprobe, digestion with RNase A and analysis of the resistant products by polyacrylamide electrophoresis in denaturing gels. Mutations are detected and localized by the presence and size of the RNA fragments generated by cleavage at the mismatches. Single nucleotide mismatches in DNA heteroduplexes are also recognized by this approach. b) Similarly mismatch chemical cleavage (MCC) provides another strategy to detect single base mismatch in DNA heteroduplexes by generic denomination (Cotton et al. 1988; Montandon et al. 1989; Gogos et al. 1990). c) The single strand conformation polymorphism (SSCP) is another method based on the differences in the secondary structure of ssDNA molecule differing in single nucleotide, which are frequently reflected in an alteration of their electrophoretic mobility (Hayashi 1990). MCC or SSCP methods can detect mutations but can not characterize them. Although the characterization of mutations can be achieved with RNase A mismatch cleavage method by using batteries of mutant riboprobes (Winter et al. 1985; Forrester et al. 1987), these methods are complicated and cumbersome. RFLP has its own limitations, which include requirements of sufficient quantity of high molecular weight DNA (50 ng) and isotopically labelled probes to obtain high level of sensitivity of detection. RFLP is also laborious as well as time consuming. PCR based methods prove to be useful and practical when nature and/or position of the mutation is known. Mutation specific PCR is the method for determining whether previously characterized mutations are present in a target sequence. The assay is based on the observation that efficient amplification under stringent PCR conditions occurs only when there is a perfect match between
the target DNA and the 3' terminus of the diagnostic primer. A single nucleotide change can thus be detected by a PCR primer having 3' terminal nucleotide complementary to the mutation. Two diagnostic PCR primers and one common primer are required for each point mutation to be tested; one diagnostic primer complementary at the 3' terminal nucleotide to the wild-type sequence, the other complementary to the mutated sequence.

In this study the procedure adopted to extract DNA (Tungpradubkul and Panyim 1985) from parasites yielded pure DNA with high molecular weight, since it was easily susceptible to restriction digestions with the various enzymes and no smear was observed in lanes loaded with uncut DNA on an agarose gel. Alu I restriction enzyme produced several smaller fragments which were seen as smear at the farther end of the gel, suggesting the presence of many Alu I sites (AG↓CT) in the *P. falciparum* genome. Like most other eukaryotic genomes, no discrete fragments are visible in Hind III, Alu I or Taq I digested total DNA on agarose gel, indicating that these enzymes have innumerable restriction sites in the falciparum DNA. It is very difficult to differentiate the smear-fragment profile of pyrimethamine sensitive and various resistant *P. falciparum* lines, more so if the point mutations are responsible for resistant phenotype.

RFLP and Southern blot hybridization have been successfully applied to detect point mutations in *ras* genes using total genomic DNA (Feinberg et al. 1983; Santos et al. 1984; Kraus et al. 1984). The genomic organization of *P. falciparum* has been studied by the Southern blot hybridization with repetitive DNA probes (Bhasin et al. 1985; Oquendo et al. 1986; Fucharoen et al. 1988). These probes have been very useful in genotyping of isolates and clones. *P. falciparum* genome contains about 10% of repetitive sequences (Hough-Evans 1982) of which more than 1% is constituted by a 21 base pair repeat (Aslund et al 1985). The complementary 21-mer oligonucleotide sequence has been employed as a probe to look for alterations in the genomic organization of pyrimethamine resistant cell lines se-
lected in vitro from sensitive F-56 parent clone. RFLP on the blots will be visible only if the point mutations create or destroy a restriction site. Comparison of band profile of Hind III digested total DNA from sensitive and various pyrimethamine resistant selected falciparum lines do not show any marked difference, prompting us to infer that most of the Hind III sites (A↓AGCTT) in the falciparum genome remain unaffected. Also Hind III digested DNA probed with 21-mer probe can not be used to detect genomic rearrangements occurring due point mutations caused by pyrimethamine pressure. However, clear differences are observed in the hybridization patterns of DNA, from sensitive and most resistant lines, digested with Alu I or Taq I suggesting that the 21-mer probe can be used to compare the genomic rearrangement of pyrimethamine sensitive and the resistant cell lines. This also indicates that Alu I and Taq I sites in the falciparum genome are definitely affected by the pyrimethamine pressure on the parasites. From these observations it can be inferred that pyrimethamine does not specifically act and affect the DHFR gene only. This method obviously can not be used for detection of point mutations in the DHFR gene using total genome, but shows alterations taking place at the Alu I and Taq I sites in the genome of pyrimethamine resistant falciparum lines. These alterations in the band profile are prominent only in the most resistant cell lines. This technique can more fruitfully be employed by first amplifying the DHFR coding sequence from the total genome and subsequently monitoring the RFLP in selected/mutated lines.

Previous studies of the effect of primer-template mismatches on mutation-specific PCR have yielded conflicting data (Wu et al. 1989; Newton et al. 1989; Ehlen and Dubeau 1989; Kwok et al. 1990; Okayama et al. 1989; Peterson 1993). Some studies revealed that certain mismatches at 3' terminus could be detected, but others reported that all the 3' mismatches produced significant decrease in the amplified product. A thorough comparison of these studies is complicated by the use of different template and primers, as well as the different reaction conditions.
specially the annealing temperatures employed. Therefore, it is necessary to optimize the annealing temperature with a set of primers in order to obtain reproducible results. Using the guidelines (Mullis et al. 1994) for primer selection and consulting the falciparum DHFR gene sequence obtained from the databank (UNDP/World Bank/WHO/TDR), the selected primers were optimized for the most stringent annealing temperature which could be employed for effective amplification and detection of resistance. These initial precautions in selecting the primers 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. The primers used are specific for detecting the pyrimethamine resistance, resulting due to base exchange at 323 base of the DHFR coding sequence. Another diagnostic primer (DR-2) which detects cycloguanil resistance failed to amplify the pyrimethamine resistant target DNA, amply demonstrating the usefulness of these primer pairs. Utility of these primers to discriminate pyrimethamine sensitive and resistant isolates from field has yet to be determined so also the RFLP studies on the PCR amplified DHFR sequence.
Figure 6.1  Ethidium bromide stained 0.8 % agarose gel containing 4 μg of DNA from clone F-56 digested with restriction enzymes and electrophoresed overnight at 30 volts

Lane 1. Undigested DNA
2. DNA digested with *Hind III*
3. DNA digested with *Alu I*
4. DNA digested with *Taq I* for 1 hour
5. DNA digested with *Taq I* for 3 hours
6. λ DNA digested with *Hind III*
**Figure 6.2** An autoradiogram of Southern blot hybridized with synthetic end labelled oligonucleotide. 4 μg of *P. falciparum* DNA from the clone F-56 and pyrimethamine selected lines digested with *Alu* I, size fractionated electrophoretically and Southern transferred on to a nylon membrane.

Lane 1. PS-5 DNA  
2. PS-4 DNA  
3. PS-3 DNA  
4. PS-2 DNA  
5. F-56 DNA
Figure 6.3 An autoradiogram of Southern blot hybridized with synthetic end labelled oligonucleotide. 4 μg of *P. falciparum* DNA from the clone F-56 and pyrimethamine selected lines digested with *Hind III*, size fractionated electrophoretically and Southern transferred on to a nylon membrane.

Lane 1. PS-5 DNA  
2. PS-4 DNA  
3. PS-3 DNA  
4. PS-2 DNA  
5. F-56 DNA
Figure 6.4 An autoradiogram of Southern blot hybridized with synthetic end labelled oligonucleotide. 4 μg of *P. falciparum* DNA from the clone F-56 and pyrimethamine selected lines digested with *Taq 1*, size fractionated electrophoretically and Southern transferred on to a nylon membrane.

Lane 1. F-56 DNA  
2. PS-1 DNA  
3. PS-2 DNA  
4. PS-3 DNA  
5. PS-4 DNA  
6. PS-5 DNA
**Figure 6.5** Sensitivity of primer pairs used for in vitro amplification of target DNA using PCR. The starting *P. falciparum* target DNA (for amplification of a DHFR segment) ranged from 0.001 to 100 ng.

Lane 1. F-56 DNA 100 ng  
Lane 2. F-56 DNA 50 ng  
Lane 3. F-56 DNA 10 ng  
Lane 4. F-56 DNA 1.0 ng  
Lane 5. F-56 DNA 0.1 ng  
Lane 6. F-56 DNA 0.01 ng  
Lane 6. F-56 DNA 0.001 ng

**Figure 6.6** Amplification of target DNA obtained from pyrimethamine sensitive clone and pyrimethamine selected *P. falciparum* lines using sensitive primer (SP) and counter primer (CP).

Lane 1. λ DNA - control  
Lane 2. blank  
Lane 3. Amplification of F-56 DNA using counter primer (CP) and distant primer (DP) - control  
Lane 4. F-56 DNA  
Lane 5. PS-1 DNA  
Lane 6. PS-2 DNA  
Lane 7. PS-3 DNA  
Lane 8. PS-4 DNA  
Lane 9. PS-5 DNA
Figure 6.7 Amplification of target DNA obtained from pyrimethamine resistant *P. falciparum* selected lines using mutation-specific pyrimethamine-resistant primer (DR-1) and counter primer (CP).

Lane 1. $\lambda$ DNA - control
Lane 2. blank
Lane 3. Amplification of F-56 DNA using counter primer (CP) and distant primer (DP) - control
Lane 4. F-56 DNA
Lane 5. PS-1 DNA
Lane 6. PS-2 DNA
Lane 7. PS-3 DNA
Lane 8. PS-4 DNA
Lane 9. PS-5 DNA

Figure 6.8 Diagnostic ability of sensitive primer (SP) and counter primer (CP) to amplify DNA only from pyrimethamine sensitive *P. falciparum*.

Lane 1. blank
Lane 2. control-1 (F-56 DNA without *Taq* polymerase)
Lane 3. $\lambda$ DNA - control, with $\lambda$ specific primer pair
Lane 4. F-56 DNA with SP and CP
Lane 5. F-56 DNA with DR-1 and CP
Lane 6. F-56 DNA with DR-2 and CP
Lane 7. F-56 DNA with SP, DR-1 and CP
Lane 8. F-56 DNA without primers, control
Lane 9. No target DNA for amplification
Lane 10. PS-5 DNA with SP and CP
Lane 11. F-56 DNA with SP only
Lane 12. F-56 DNA with CP only
Lane 13. $\lambda$ DNA with SP and CP
Lane 14. Human DNA with SP and CP
REFERENCES


Bhasin V.K. and Trager W. (1987) Gametocytocidal effect in vitro of cinchona alka-


Foote S.J., Galatis D.and Cowman A.F. (1990) Amino acids in the dihydrofolate re-


Giemsas G. and Werner H. (1914) Erfahrungen mit weiteren dem chinin mahestehenden Alkaloiden und einiger ihrer Derivate bei Malaria. \textit{Archiv Fiir Schiff's und Tropenhygiene} 18: Beiheft 5.


Maberti S. (1960) Desarrollo de resistencia a la pirimetamina. Presentacion de 15


of cis-dichlorodiammine platinum (II) and related transition metal complexes on *E.coli*. Antimicrob.


nucleic acid synthesis in *Plasmodium gallinaceum* and *Plasmodium berghei*. Biochem. Pharmacol., 6, 143-152.


TDR news (1994) UNDP/World Bank/WHO, March, no. 44


