GENETIC STUDIES OF MALARIA PARASITES, ESPECIALLY IN RELATION TO DRUG RESISTANCE

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SUMMARY

1. A chloroquine-resistant parasite line of Plasmodium chabaudi was obtained by submitting a sensitive line to a continuous and gradually increasing level of drug-pressure. The line 411AS survived treatment of 3 mg/kg given for 6 days which eliminates the original drug sensitive line.

2. Cyclical transmission of 411AS as well as stability studies carried out showed that resistance was stable and transmissible through mosquitoes in the absence of drug pressure.

3. The genetic basis of chloroquine resistance was studied by making a cross between 411AS and a sensitive line of different origin, 96AJ, which differed additionally in 3 characters. Seventy clones from this cross were classified. Various recombinant classes were obtained and the results showed that chloroquine resistance in P. chabaudi is a stable character which undergoes genetic recombination with other markers.

4. Another line, 524AJ, resistant to 3 mg of chloroquine/kg administered for 6 days, was obtained by similar low drug pressure method.

5. Cyclical transmission of 524AJ and from clones established from this population showed that this line exhibited two types of resistance: (a) a stable, heritable form detectable after mosquito transmission, and (b) an unstable form which could not be detected after mosquito transmission.

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6. Competition studies between chloroquine-resistant and chloroquine-sensitive parasites were made by mixing different proportions of blood forms in mice and by establishing and testing clones at different stages of the infection. Sporozoites from mosquitoes which fed on each mixture were also used to establish new infections in mice and clones were established and tested in a similar way. All the experiments showed an apparent selective advantage of the resistant over the sensitive forms in the absence of chloroquine pressure.

7. A discussion of the selection experiments, crossing experiments and competition studies is included and further research suggested by this work is considered.

1. Introduction

History of man is made of several descriptions of war, with different civilizations having in common the ambition of spreading their frontiers and cultures, and dominating larger areas of land. Such processes are always costly to both sides, despite, no doubt, some advantages in the short term, to the winner. In peace time, countries have tried to develop and improve living conditions so that disease, morbidity and mortality can be reduced in the population. However, it was only in 1948 that the United Nations presented a co-operative programme in which countries from developed and under-developed areas could prevent, by Public Health schemes, infections from spreading as epidemics, these programmes being administered by the newly created World Health Organization.

As malaria was one of the most devastating illnesses of all times (Livingstone, 1971) its eradication was planned making use of insecticides against the mosquito vector and antimalarial drugs against the parasite. Antimalarial campaigns met success in countries such as Sri Lanka (formerly Ceylon), Mauritius and Cyprus and to a lesser extent in India. But, counterbalancing the improvement of Public Health in many countries, two major problems appeared: the development of drug resistant malaria parasites in some areas of South America and South East Asia, and the beginning of the Vietnam War which affected several countries from different continents, involving increased movements of human populations. Reports on the emergence of parasites resistant to drug treatment encouraged the production of new drugs and research on the origin and mechanisms of resistance. It is important to mention here that intelligent use of any drug, either for treatment

or prophylactic measures is not always feasible, since many areas are occupied by migratory populations and the Health Services in developing countries may be poorly equipped and staffed. The effect of malaria on American soldiers in Vietnam, in which the number of soldiers evacuated from action due to malaria was as high as due to wounds (Tigert, 1966), stimulated research on malaria therapy as well as resistance to malaria drugs mainly related to chloroquine, a drug widely used and wrongly conceived as incapable of causing resistance (Schmidt, 1969). Chloroquine was used as a substitute for quinine since quinine synthesis was uneconomical (Davey, 1951) and the world natural supply of quinine was lost to the Allies during the Second World War. The approach which has been taken in this work is best summarized by a quotation from Sir Charles Harington, who, in 1957, concisely remarked that ... "however hard and successfully we may work in the search for new drugs we shall therefore continue to labour under discouragement so long as we are faced with the bug bear of drug resistance. The problem is one of microbial biochemistry, physiology and genetics and can only be solved by work in these fields".

The purpose of this project is to investigate the genetic factors involved in chloroquine resistance in malaria. Drug resistance can be determined by chromosomal changes and cytoplasmic factors. In bacteria, some drug resistance is associated with episomes or R factors which are cytoplasmic (Watanabe, 1963; Watanabe and Fukasawa, 1966). Some classes of erythromycin-resistance have been found to be determined by cytoplasmic genetic factors located in the mitochondria in *Paramecium aurelia* (Beale, 1969). The development of genetically stable pyrimethamine-resistant mutants of *Plasmodium chabaudi* (Walliker *et al.*,

1975) as well as stable metachloridine resistance induced in P. gallinaceum (Bishop, 1958b) suggested chromosomal changes (Walliker, 1976). Drug resistance of Protozoa has been extensively reviewed by Bishop (1958a) and Schnitzer (1963) with the description of methods used with trypanosomes and malaria parasites and the results obtained. Information on the origin of such resistance and the location of the altered genes, if any, can only be carried out by analysing the progeny of resistant parasites in the absence of drug pressure, by making use of specific chemicals which interfere with plasmids that may carry the resistance character, and by crossing resistant parasites with parasites of different origins, simultaneously comparing the reciprocal cross results.

In this work the problem of chloroquine resistance has been investigated using rodent malaria parasites both because of genetic work which has already been carried out using these forms and because they represent a convenient model for comparative studies with human malaria.

1.1 Concept of drug resistance.

Tolerance resulting from prolonged use of a drug is historically exemplified by the story of Mithridates (B.C.) a king who, in order to protect himself from the effects of poisons had a lifelong habit of taking such substances prophylactically, which rendered him unable to take his life by poison when defeated in battle, (Bishop, 1958a). Early studies on resistance to drugs in micro-organisms were made using bacteria, trypanosomes and malaria parasites. In 1910, Ehrlich predicted that trypanosomes would develop resistance to any type of

drug designed to attack them, thus suggesting that organisms may possess mechanisms which permit them to survive under normally lethal conditions.

When resistance to chloroquine and to other drugs arose in malaria parasites, it became necessary to define drug resistance in a precise way, in order that similar tests could distinguish different types or levels of resistance in different areas. The W.H.O. (Geneva, 1973), defines resistance as a "drug-parasite interaction in which there is the ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal or higher than those usually recommended but within the limits of tolerance of the subject". In general terms, it is a temporary or permanent loss of the initial sensitivity of the micro-organism to the effect of the active substance. This loss of sensitivity should not be confused with the absence or inadequacy of drug action due to deficient absorption by the host, unusual rate of degradation or excretion of the drug or failure of the host to take the drug, which are host factors which may complicate the recognition of resistance.

1.2 Chloroquine resistance in human malaria

The first reports confirming that normal doses of chloroquine had failed to cure an infection with P. falciparum came from Colombia in 1961 (Moore and Lanier; Young and Moore) although in 1960, in Venezuela, Maberti had already expressed suspicion on the effectiveness of treatment with chloroquine in another human malaria infection (cited by Peters, 1970). However, publications long before 1961 warned against the possibility of resistance. Most et al. (1946) and Earle and Berliner (1948) cautioned against the use of doses which were

effective for P. vivax being administered against P. falciparum strains which might not have been so sensitive to the drug. Resistance of malaria parasites to chloroquine did not appear for more than a decade after its inception for antimalarial therapy. Resistance to this drug was thus slow to emerge, when compared to other drugs such as proguanil, to which resistance was detected four years after its initial use, and pyrimethamine, to which resistance was reported after only two years (Peters, 1970). From 1961, a large number of cases of malaria resistant to chloroquine were recorded. After further South American reports (Moore and Lanier, 1961, from Colombia; da Silva et al. 1961; Rodrigues, 1961, from Brazil; Godoy et al., 1975, from Venezuela) a number of instances of lack of response of P. falciparum to treatment with chloroquine in South East Asia were reported (Harisanuta, 1962; Montgomery and Eyles, 1963; Eyles et al., 1963; Powell et al., 1964; Sandoshan et al., 1966 and Ebisawa and Fukuyama, 1975). Several claims have been made for the existence of chloroquine resistance in Africa, but many of these reports have subsequently been shown to be false (Jeffery and Gibson, 1966) due to the inadequacy of the tests carried out and the results reported (Stevenson, 1966; Lasch et al., 1965; Bruce-Chwatt, 1974). More recently, a careful study on chloroquine tolerance by Dennis et al. (1974) on a strain from Ethiopia and a case reported by Pillay and Bhoola (1975) on a P. falciparum strain not responding to the drug, in South East Africa, give circumstantial evidence on the possible existence of resistance to the drug in Africa.

It is of interest that of the four species of human malaria parasites, resistance to treatment with chloroquine concerns only P. falciparum. This may be partly due to the greater number of studies

on this species because of its pathogenic importance, but it is also possible that specific characteristics may be involved in the development of chloroquine resistance.

1.3 Chloroquine resistance in laboratory malaria parasites

The first report of chloroquine-resistance in a laboratory malaria parasite was in 1956 when Ray and Sharma reported lack of response to chloroquine in the avian species P. gallinaceum. Following treatment with a low initial drug dose and prolonged passaging under drug pressure, the parasites became twice as resistant as the initial parental strain. In 1957, Ramakrishnam et al., using a similar technique with P. berghei induced drug-resistance of 200 fold magnitude, after 33 blood passages. Before these two reports, other research had been carried out with the intention of producing chloroquine resistance in various malaria species although unsuccessfully. These projects included research on avian malaria parasites such as P. lophurae (Thompson et al., 1948), P. gallinaceum (Seaton, 1951; Bishop and McConnachie, 1952) and later in P. relictum (Kollert, 1963). Simian malaria parasites were also used, including P. knowlesi and P. coatneyi (mentioned in WHO report, 1967).

Numerous studies have now been made on chloroquine-resistance in rodent malaria species. The situation appears to be complicated, as there is a natural variation in resistance to the drug among isolates of the various species, and examples of both stable and unstable resistance have been reported. Studies carried out on each of the four species can be summarized as follows:

(1) P. yoelii - All isolates of this species so far examined exhibit an innate resistance to chloroquine. This is found in newly isolated

parasite lines which have not previously encountered the drug (Warhurst and Killick-Kendrick, 1967; Peters, 1968; Carter, 1972).

(2) P. berghei - Isolates of this species are naturally chloroquine-sensitive but resistance has been produced by several authors and the techniques employed varied significantly. Ramakrishnam et al. (1957), Kollert (1963), Peters (1965) and Jacobs (1965) employed similar techniques, using low drug doses and frequent blood passaging of parasites surviving drug pressure for the selection of resistant lines. High doses of chloroquine were used by Sautet et al. (1959) and Benazet (1965) in a relapse method in which passaging was carried out about ten days after infection of mice. Hawking and Gammage (1962) induced resistance in P. berghei by blockage of the immune system of the host and by drug administration in the diet. Although successful once, the same method failed when applied to four other lines (Warhurst, 1965). A rapid method was attempted by Hawking (1966) in which a single high dose of chloroquine was administered to mice with high parasitaemias. One and five hours after administration of 50mg of the drug, the blood of these animals was inoculated into uninfected mice and the whole process repeated. In neither case, however, were resistant parasites produced due, probably, to the fact that the peak of chloroquine concentration in the plasma is only achieved four hours after the administration of drug (Macomber et al., 1966). A feature of chloroquine resistance in P. berghei is its instability in the absence of drug-pressure. For example, Peters (1965) developed a highly resistant strain (RC), but the resistance was lost on removal of the drug. Reports of the occurence of stable chloroquine resistance in P. berghei (e.g. Peters

et al., 1969) may be due to selection of P. yoelii from mixed infections of P. berghei and P. yoelii, the morphology of the blood forms of these two species being very similar.

(3) P. vinckei - Only one successful attempt to produce chloroquine-resistance in this species has been reported. Powers et al. (1969) used a low pressure method to induce resistance in a strain which was already resistant to pyrimethamine.

(4) P. chabaudi - Until the present work no chloroquine-resistance has been reported in this species. Peters (1970) showed that a line of P. chabaudi possessed similar sensitivity to P. berghei.

1.4 Chloroquine- mode of action and mechanisms of resistance

Chloroquine acts as a schizonticidal drug, being active mainly against the asexual erythrocytic forms of the parasites. Despite numerous studies by several workers, its precise mode of action remains unclear and explanations for resistance to the drug are largely speculative.

Several studies, making use of bacterial nucleic acids, have demonstrated the interaction of chloroquine with DNA. Hahn et al. (1966) reported on the similarity of quinine and chloroquine on the formation of complexes with double stranded DNA with consequent blockage of DNA and RNA synthesis. This work was carried out with Bacterium megaterium, and ribosome degradation as well as inhibition of protein synthesis are also described. Cohen and Yielding (1965) reported on the intercalation of chloroquine with DNA base pairs. Stollar and Levine (1963) reported on the inhibition of bacterial transformation by chloroquine, which binds to cytosine and guanine, and Allison et al. (1965) described changes of the double stranded DNA

helix which is stabilized by the drug at low concentrations. Results from research on bacterial DNA might not be applicable to Plasmodia since DNA in Protozoa is attached to proteins such as histones. Bahr and Mikel (1972) with very interesting work on the arrangement of DNA in the nucleus of P. berghei, P. chabaudi and P. vinckei (rodent malaria parasites) reported on the similarity of host and parasite chromatin structure and composition. These factors, together with the presence of the nuclear membrane at all stages of the growing trophozoite, are unfavourable to the hypothesis of the nucleus being the main target of chloroquine activity.

From a wide range of information on the effects of chloroquine on the malaria parasite it appears that the accumulation of the drug within the red blood cells is dependent upon differences between the pH of the plasma and the pH within the red blood cell (Rollo, 1969). Macomber et al. (1966) suggested that susceptibility to chloroquine was due to permeability to the drug and accumulation of a critical concentration. These studies were carried out with red blood cells parasitized with resistant and sensitive P. berghei, the latter concentrating twice as much drug. Even greater concentrations of chloroquine in red blood cells containing sensitive malaria parasites were demonstrated with more accurate techniques by Fitch (1969, 1972), who considered that such accumulation was dependent on the existence of binding sites in the malaria cell. Bodammer and Bahr (1973) referred to the existence of a modified area of the red cell, caused by the parasite; a site closely attached to the red cell membrane which is presumed to be related to the transport of metabolites and which is perhaps a main target of antimalarial activity.

Reaction of the drug in the cell is associated with a progressive change in the pH of the digestive vacuoles as the acid radicals of the parasite proteolytic enzymes are buffered by basic chloroquine ions, and the parasite is unable to digest haemoglobin. The lysosomal organelles of mammalian cells which are at acid pH and in which the drug is found to be concentrated are considered to be analogous to the digestive vacuoles of the malaria parasites (Homewood et al., 1972). Howells et al. (1972) using P. berghei proposed that chloroquine induced starvation of the parasite by causing clumping of the pigment and autophagosome formation. In vivo and in vitro studies proved the necessity of active haemoglobin metabolism and the presence of the digestive vacuoles for chloroquine action. Sporogonic and exoerythrocytic forms of malaria parasites as well as mature merozoites, which contained no pigment or haemoglobin metabolism or digestive vacuoles, were unaffected by the drug (Macomber and Sprinz, 1967). Apart from the amino acid deprivation due to direct change on the pH of the malaria cell, chloroquine may also labilize phagosome membranes with consequent leakage of enzymes resulting in cytoplasmic destruction (autolysis) (Peters, 1974).

Theories concerning the mechanism of resistance to chloroquine have been related to physiological alterations detectable in resistant strains of P. berghei and P. falciparum. The decrease in chloroquine uptake of red blood cells parasitized with resistant malaria parasites (Macomber et al., 1966) was suggested to be due to a deficiency of drug-binding sites in the parasites (Fitch, 1972). A different theory was proposed by Howells (1970) who suggested a change to aerobic metabolism in the respiratory mechanism of P. berghei resistant to

chloroquine. However, this theory has now been questioned because of difficulties in the technique used for the detection of functional mitochondria and because of host-cell contamination (Howells and Maxwell, 1973a, b).

Thus, the precise way in which chloroquine acts on sensitive forms and the mechanism involved in resistance are still unclear. Because of the various types of resistance seen among the rodent species (described above in Section 1.3), it is possible that several mechanisms may be involved.

1.5 Genetics of malaria parasites

Studies on the genetics of malaria are technically difficult because of the parasite's complex life cycle. Furthermore, dramatic morphological changes suggest considerable variation in gene expression. The number of chromosomes has not yet been determined and little information is available on the location of genes and on the possible existence of genetic material in the cytoplasm. Genetic studies on malaria parasites are important for a complete understanding of phenomena such as drug resistance, its origin and dissemination in parasite populations.

Greenberg and Trembley were the first to attempt to cross lines of Plasmodia, using two lines of P. gallinaceum differing in their response to pyrimethamine and in their ability to produce exo-erythrocytic schizonts. The first crosses were unsuccessful (Greenberg and Trembley, 1954,a; Trembley and Greenberg, 1954) as no evidence of hybridisation was found. In a later experiment (Greenberg and Trembley, 1954,b), indications of recombination between the characters distinguishing the parent lines were reported. This interpretation

was, however, uncertain since one of the genetic markers (production of secondary exo-erythrocytic forms) was known to be unstable. In 1971, Walliker et al. demonstrated genetic recombination in rodent malaria parasites using two lines of P. yoelii which differed in their response to treatment with pyrimethamine and in the forms of the enzyme glucose phosphate isomerase (GPI). A similar experiment was carried out with P. chabaudi (Walliker et al., 1975), crosses being made between two lines differing by two enzyme markers (6PGD and LDH) as well as by sensitivity to pyrimethamine. These experiments showed that recombination and segregation of the parental enzyme forms had occured before the emergence of parasites in the blood, results which showed that the blood forms were haploid. Cytological evidence suggested that a reduction division occured probably during development of the oocyst (Sinden and Canning, 1973). More recent studies were carried out with lines of P. yoelii differing in the response to treatment with pyrimethamine, enzyme forms (GPI) and in the growth pattern of each line (virulent or mild course of infection). These studies (Walliker et al., 1976) showed that variations in virulence were due to genetic differences between the two parental lines.

In 1969, Yoeli et al. reported the possible existence of a gene transfer mechanism between malaria parasites called "synpholia" which may occur between two parasites developing in the same red blood cell. However, confirmation of this process has not, so far, been made.

The genetic uniformity of the parasite population within lines, for the purpose of a cross, as well as the stability of the characteristics used as genetic markers is of great importance. Carter's (1973) identification of enzyme types from different rodent malaria

parasites facilitate the use of these laboratory models for genetic studies. Utilization of a chloroquine resistant line in a cross with a sensitive line had not yet been carried out but it was highly relevant to use such a marker in order to understand the genetic basis of chloroquine resistance.

1.6 Objective of this project

The object of this project has been to investigate the origin and genetic basis of resistance to chloroquine in the rodent malaria species P. chabaudi. In the past, few studies on drug resistance have been made using this species; for genetic work, however, it is of considerable value because of the extensive amount of enzyme variation found among wild isolates (Carter and Walliker, 1975) which can be used for recombination studies in crosses (Walliker et al., 1975).

In the first section a description is given of attempts to produce chloroquine resistant lines of P. chabaudi using a variety of drug selection methods. Once a line resistant to chloroquine was selected, studies on the stability of resistance in the absence of drug pressure were carried out.

The genetic basis of the resistance obtained in one line was then investigated in a cross with a sensitive line of different origin, the products of the cross being analysed for each of the parental markers.

In the final section, competition studies involving mixtures of resistant and sensitive parasites are described in order to investigate the fate of the resistant parasites in a mixed population and in the absence of drug pressure. Such studies were made on sporozoite-induced as well as on blood form transmitted infections.

2. Material and Methods

2.1 Lines of Plasmodium chabaudi

2.1.1 Origin of Plasmodium chabaudi

P. chabaudi was first described by Landau (1965) who isolated the parasite from thicket rats (Thamnomys rutilans, Peters,1878) of the Central African Republic. The development and infection pattern of the parasite were studied by Landau and Killick-Kendrick (1966), Wery (1968) and Landau et al. (1970). Bafort (1968) reclassified the parasite as a sub-species of P. vinckei, but due to later studies on the enzyme forms and morphology of the parasite (Carter and Walliker, 1975), the species has been renamed P. chabaudi.

2.1.2 Life-cycle

P. chabaudi is a typical mammalian malaria parasite with a life-cycle which involves an asexual phase in the mammalian host and a sexual phase in the insect vector. In the vertebrate host there are two cycles of division, one termed exo-erythrocytic schizogony in the parenchyma cells of the liver and the second, erythrocytic schizogony, in the blood. The infection commences when uninucleate sporozoites are injected into the blood stream by female mosquitoes. In less than an hour, by an unknown process, liver parenchyma cells are invaded and each sporozoite develops by nuclear division into a schizont which matures in 52 - 53 hours producing a large number of merozoites, which are released into the blood.

In many mammalian malaria parasites, secondary exo-erythrocytic cycles of development may also originate from these merozoites, although it is not yet known whether such cycles occur in P. chabaudi. In the blood, merozoites invade mature erythrocytes, each parasite

forming a trophozoite which grows and divides to produce a schizont containing, usually, 4 - 8 merozoites which are released by rupture of the host cell. Some merozoites reinvade further red blood cells and the same cycle is repeated. The cycle takes 24 hours, and development of schizonts is synchronous, schizogony normally occurring around midnight. Sexual forms, termed macro- and micro-gametocytes, are produced by some merozoites, which usually predominate after the peak of the infection (from the tenth day of infection onwards).

The complete development of gametocytes takes place in the mosquito host in which fertilization between micro- and macro-gametes occurs. The zygote is termed an ookinete, which penetrates the midgut wall of the mosquito, to produce an oocyst. The first divisions of the nucleus of the zygote are thought to be meiotic (Sinden and Canning,1973). Eventually, nuclear divisions result in the formation of several thousand sporozoites which can be seen in the salivary glands of the mosquitoes from the 11th day after infection with P. chabaudi. The sporozoite is inoculated into the new host to recommence the life-cycle.

2.1.3 Definition of terms

It is essential to describe the terms "isolate", "line" and "clone", which will be used frequently. These terms will be taken to have the following meanings:-

(i) Isolate - this refers to a sample of parasites collected on a single occasion from a wild rodent or mosquito and preserved either by passaging through laboratory animals or kept as deep-frozen material. An isolate is not necessarily genetically homogeneous and may even contain representatives of more than one species.

(ii) Line - this refers to a collection of parasites which have undergone a particular laboratory passage. By strict definition every laboratory manipulation of parasites creates a new line, but usually parasites are described as belonging to a line only after a special treatment, such as selection for drug resistance. All the parasites in a "line" have certain characteristics in common but they need not be genetically identical.

(iii) Clone - a clone is a group of genetically identical organisms derived from a single cell by asexual reproduction. Cloning is a procedure of particular importance in genetic work with unicellular organisms, and should always be carried out, if feasible, prior to determining the characteristics of a new isolate, before using an isolate in genetic experiments, and again before classifying the progeny of a cross.

(iv) Stabilate - this refers to a population of an organism preserved in a viable condition on a unique occasion (Lumsden and Hardy, 1965) such as liquid nitrogen preservation of a P. chabaudi line.

2.2 Host species used in this project (Table 1)

For the vertebrate host of P. chabaudi mice and white rats were used. The rodents were housed in propylene cages using sawdust as bedding, fed with animal cake (MacGregor's) and supplied with drinking water supplemented with 0.05% PABA (Para-aminobenzoic acid) an essential growth requirement for the parasite (Hawking, 1953; Peters, 1970). The animal room was maintained at 18 - 22^{o}C under natural light conditions. For precise drug resistance and selection tests, female

C57 Black mice, aged 4 - 5 weeks were used; for routine passaging and cloning C57 Black mice and a selection of mice of mixed strains were used. For cyclical transmission, mosquitoes (Anopheles stephensi) were used which were maintained at 25°C and 90% humidity conditions with alternating 12 hour sequences of light and darkness. They were fed with a 10% solution of glucose in a 0.05% solution of PABA in water.

TABLE 1 List of rodents and insect host species.

Host Species	Source
Vertebrate:	
Mouse (Mus musculus):	
C57 Black, inbred	Centre for Laboratory Animals Edinburgh
Mixed strains	Department of Genetics Mouse House, Edinburgh
White rat (Rattus norvegicus)	Centre for Laboratory Animals Edinburgh.
Invertebrate:	
Mosquito (Anopheles stephensi)	Laboratory colony.

2.3 Maintenance of malaria parasites in the laboratory

2.3.1 Blood passage

Infections were maintained by passaging parasitized red blood cells from infected rodents into uninfected animals by injection of infected blood diluted either in citrate saline (0.9% NaCl, 1.5% Na citrate) or in heparinised serum/Ringer (50% calf serum, 50% mammalian Ringer). Inoculation was carried out by intravenous or intraperitoneal routes.

2.3.2 Cyclical transmission

Mosquito transmission of malaria parasites was based on the method described by Landau and Killick-Kendrick (1966). Infected rodents, in which gametocytes were present, were exposed to mosquitoes which had been starved for 24 - 48 hours. The infected rodents were exposed to mosquitoes for a varied length of time (1 - 6 hours) depending on the feeding performance of the mosquitoes. Seven to ten days later a small number of mosquitoes were dissected in order to count the number of oocysts which had developed on each midgut. Fifteen and, eventually, seventeen days after the blood meal, when sporozoites were present in the salivary glands, an uninfected mouse was exposed to the mosquitoes for transmission of the malaria infection. Patent blood infections could be detected after 4 - 8 days.

2.3.3 Liquid nitrogen preservation (Deep-freezing)

When parasite lines were not being maintained by animal passage, infected blood was stored in sealed capillaried (stabilates) kept in liquid nitrogen, using the method of Lumsden et al. (1966). Such material, inoculated intraperitoneally into a rodent, after any period of storage, induced an infection which became patent from 7 - 14 days after inoculation.

2.4 Parasitaemia estimation

A thin blood smear was prepared, fixed in methanol and stained with Giemsa's stain at pH 7.2. Blood was not forced out of the tail as parasites adhering to blood vessel walls might have distorted the proportion of parasitized red blood cells present in the total number of red blood cells counted. The sample size varied from 10,000 red

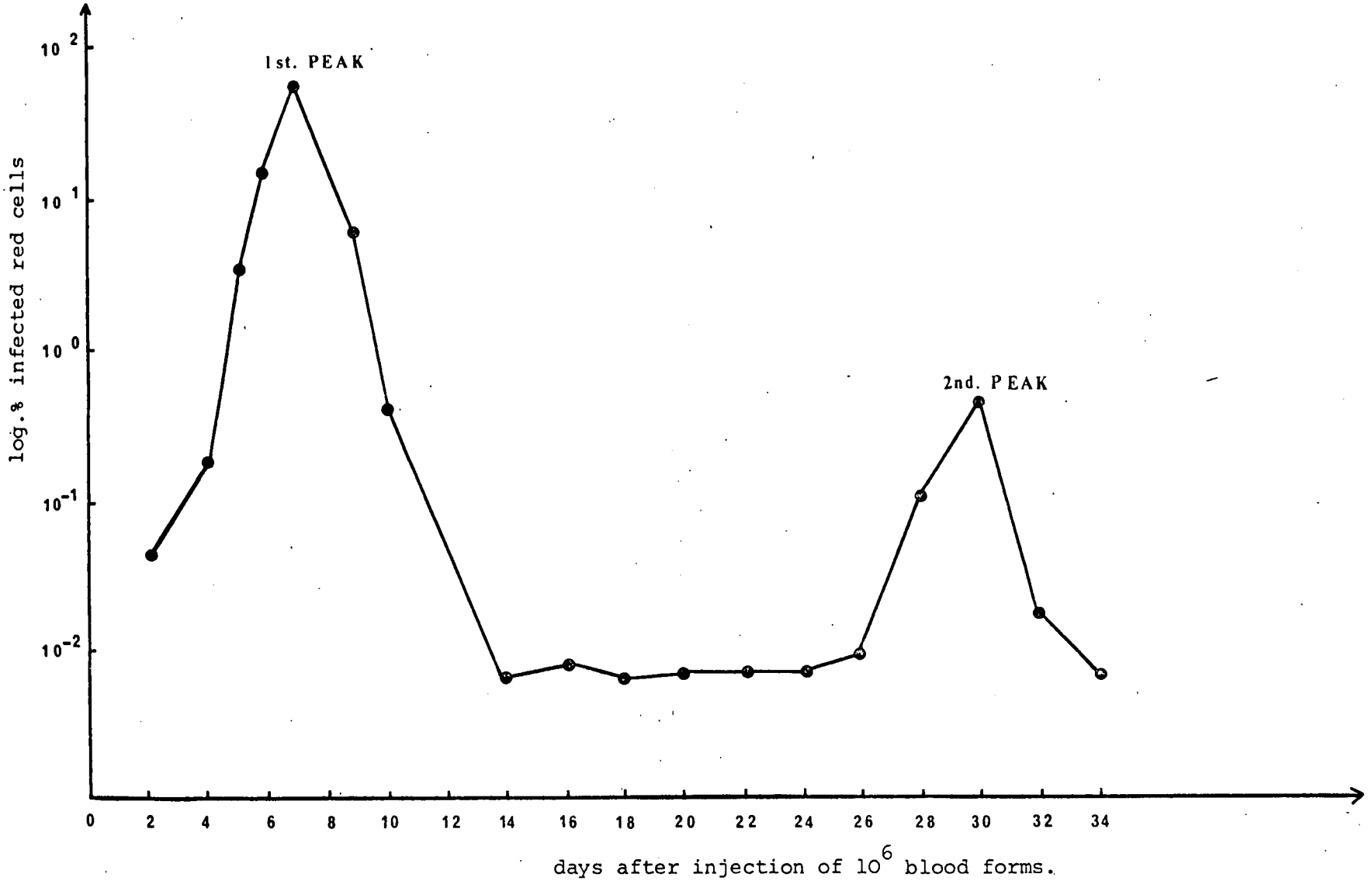


Figure 1. Typical parasitaemia curve of a P. chabaudi infection.

blood cells counted when parasitaemias were low, to 1,000 for parasitaemias higher than 5%.

2.5 Preparation of standardized inocula of malaria parasites

Inocula containing known numbers of parasites were required for drug resistance tests and for establishing clones by dilution of infected blood. Counts of red blood cells of the donor mouse were carried out using a Coulter counter, and the parasitaemia was calculated. The number of parasites present in a given volume of blood could then be determined.

2.5.1 Inocula containing 10^6 parasitized red blood cells

A donor was anaesthesized with ether and bled from the brachial vessels, the blood being collected with a Pasteur pipette containing a drop of heparin. The blood was diluted in serum: Ringer so that 10^6 parasites were present in 0.1ml volume of diluent. Inoculation of mice with 0.1ml aliquots was carried out not later than an hour after bleeding the donor. At all stages diluted blood was kept on ice.

Figure 1 represents a typical parasitaemia of a *P. chabaudi* infection in mice inoculated with 10^6 blood forms.

2.5.2 Cloning technique

To establish clones of blood forms of *P. chabaudi* in mice, a dilution technique was employed (Walliker, 1976). Dilutions were made from a donor mouse in which at least 90% of the infected erythrocytes contained single parasites. Mice with low parasitaemias, early in the infection, were generally most suitable. The procedure for cloning by dilution was as follows: a 5 µl sample of blood was

collected from the tail of the donor rodent and diluted in 1 ml of a serum: Ringer solution. Further dilution steps were then made until a concentration of 0.5 parasite per 0.1 ml of inoculum was obtained. 0.1 ml inocula were then injected into a group of 30 - 40 mice. Using this dilution approximately 40% of the animals injected became infected, of which approximately 75% were predicted to contain infections derived from single parasites.

2.6 Source, preparation and administration of drugs

2.6.1 Chloroquine

Chloroquine was obtained from May & Baker Ltd. (Dagenham, England) in the form of "Nivaquin", a solution of chloroquine sulphate (40 mg chloroquine base per ml). For treatment of mice, dilutions of this solution were made in distilled water. Drug dose was expressed as mg of chloroquine per kg of mouse body weight, so that for mice weighing 10.0 gm, 0.1 ml of drug solution administered 3 mg of chloroquine base concentration. The drug was administered orally, by intubation, in 0.1 - 0.2 ml amounts. Drug solutions were kept under refrigerated and dark conditions.

2.6.2 Pyrimethamine

Pyrimethamine was obtained from the Wellcome Research Laboratories (England) in the form of a powdered base ("Daraprim"). The drug was made up as a suspension ~~using a weak solution of acetic acid and~~ in distilled water and further dilution from this stock was made with a 2% solution of carboxymethyl cellulose ~~(ratio 1:5).~~ according to the method of Yoeli et al (1969) Drug solutions were kept under refrigerated conditions and thoroughly shaken on a Vortex mixer before administration. The drug was administered orally to mice in 0.1 - 0.2 ml amounts.

2.7 Enzyme characterization by starch gel electrophoresis

Electrophoretic variants of the enzyme lactate dehydrogenase (LDH) and 6-phosphogluconate dehydrogenase (6PGD) were examined using the method of Carter (1973). Parasitized blood was collected from two mice at the peak of the infection by bleeding from the brachial vessels into citrate saline. After centrifugation at 1,500g for 5 minutes, parasites were released from their host cells by incubation of the pelleted blood cells with half their packed volume of a 0.15% saponin solution in normal saline at 37°C for 20 minutes. The freed parasites were then separated from red cell stroma by high speed centrifugation (4,000g for 10 minutes) after the addition of Ringer solution, and transferred to a glass container stoppered with non-absorbent cotton wool for freeze-drying. On completion of the freeze-drying process, containers were sealed and kept, under vacuum, at -20°C. For electrophoresis, 2mg of the freeze-dried material was dissolved in 0.02ml of distilled water. The gel buffer was 0.1M tris-HCl, pH 8.5 for lactate dehydrogenase and 0.1M tris-HCl, pH 7.0 for 6-phosphogluconate dehydrogenase.

2.8 In vitro culture

An in vitro culture system, based on Rieckman's technique for P. falciparum (Rieckman et al., 1968; Rieckman, 1971) was designed for P. chabaudi, as follows: 3 - 4 days prior to the test, 30 - 40 mice were injected with a high number of parasites (1-2 x 10^7 parasites). When the average parasitaemia was approximately 2% all mice were bled into a conical flask containing heparin, (5 i.u. per ml. blood) and kept on ice. Bleeding from the brachial vessels was carried out in aseptic conditions. 1 ml aliquots of the blood were then placed into screw cap, flat bottomed glass vials

(length: 9.0 cm, width: 3.0 cm, thickness: 0.3 cm) each of which contained glucose (5 mg) and either no chloroquine (controls) or chloroquine added in various quantities (0.2 mg, 0.4 mg, 0.8 mg and 1.0 mg). Each vial contained 200 units of penicillin solution. The blood was shaken gently and the vials were placed in a water bath at 39° - 41°C for a period of 6 hours. Incubation started at about 9 p.m. and blood smears were made from each sample at 10 p.m., 11 p.m., 12.30 a.m. and 1.30 a.m. Vials were slightly shaken before smears were taken.

2.9 Selection of a line of P. chabaudi resistant to chloroquine

2.9.1 Origin of parasite material and phenotype

Two lines of P. chabaudi were used in this work. One line, denoted P. chabaudi AJ from the isolate AJ (Carter, 1973) was characterized by an electrophoretic form of the enzyme 6 phosphogluconate dehydrogenase denoted 6 PGD-3, a form of lactate dehydrogenase denoted LDH-2 and was sensitive to pyrimethamine. The other line, denoted P. chabaudi AS from the isolate AS was characterized by an electrophoretic form of the enzyme 6-phosphogluconate dehydrogenase denoted 6 PGD-2, a form of lactate dehydrogenase denoted LDH-3, and was resistant to 4 daily doses of pyrimethamine at 15mg/kg (Walliker et al., 1975).

2.9.2 Methods used for selection of chloroquine resistant mutants

Two methods were used in attempts to select mutants resistant to chloroquine:

(i) High pressure method:

This method, carried out only with line AJ, consisted of infecting mice by inoculation of 10^6 parasites followed by

treatment of the mice with high doses of chloroquine for 4 consecutive days, starting four days after inoculation of parasites. Separate tests were carried out using 20 mg, 40 mg and 60 mg of chloroquine/kg/day. Mice were examined for infection for 15 days following completion of drug treatment. If relapses occurred parasites were passaged into a new set of mice and treatment was repeated to test for resistance.

(ii) Continuous low pressure method:

This method was used for both lines AJ and AS. 10^6 parasites were injected intraperitoneally into two groups of mice, one group being submitted to a low dose of chloroquine administered 3 hours after inoculation and at a similar time on subsequent days and the second group being kept undrugged. Sub-passages of infected blood were made weekly from the mouse exhibiting the highest parasitaemia, except in instances where slow development of the parasite required some extension of this period. The course of infection was followed by examining blood smears, prepared every 48 hours for each mouse, commencing 24 hours after inoculation. The number of drugging doses was established from the growth of parasitaemia in treated mice and drug pressure was gradually increased for each passage according to the growth of infection in the preceding passage. For transmission of malaria parasites through mosquitoes, drug pressure was maintained continuously until gametocytes were observed, usually on day 11 - 13 after inoculation. The infected mouse was then exposed to mosquitoes.

By counting parasitaemias for each mouse within group a ratio R was calculated:

$$R = \frac{\text{mean parasitaemia (\%) in drugged group}}{\text{mean parasitaemia (\%) in undrugged controls}} \times 100$$

This R ratio permitted comparisons to be made between experiments carried out at different times during the year. Control studies with a sensitive line were carried out simultaneously and under identical conditions.

2.9.3 Standard test for chloroquine resistance

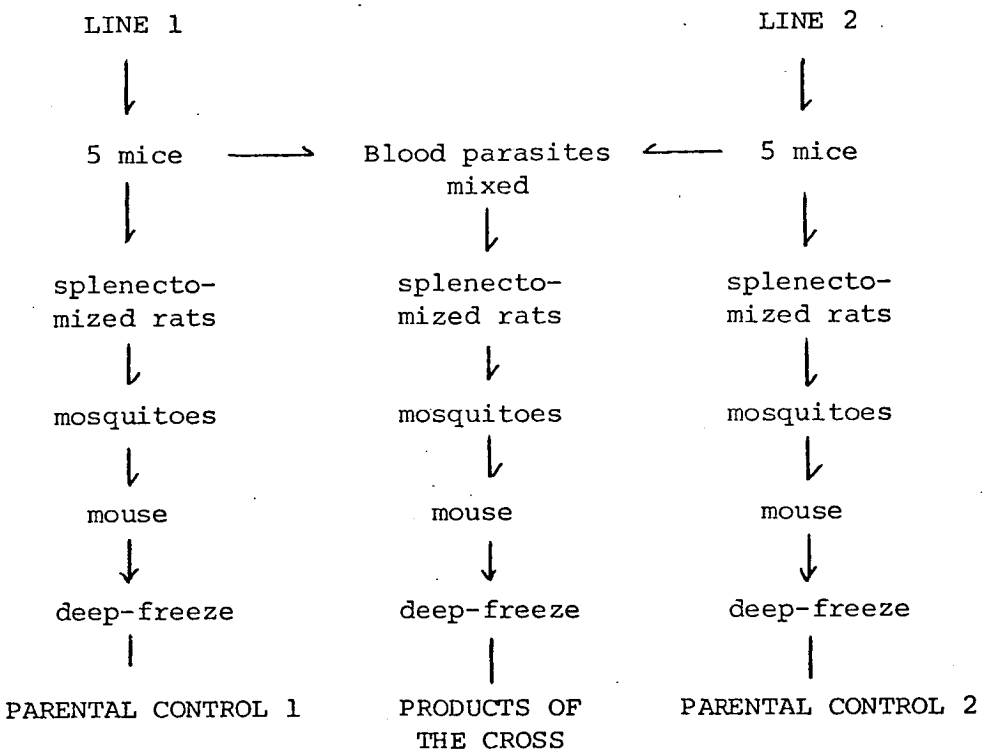
Following the production of chloroquine resistance in *P. chabaudi* a standard drug test was devised to distinguish between resistant and sensitive forms. 10^6 blood forms were injected into mice which were treated with chloroquine at 3 mg/kg daily for six days, the first dose being given 3 hours after injection of parasites. On day 4 after inoculation of parasites, mice were individually reweighed and the drug volume given was readjusted to the increase recorded. Blood smears were examined on the sixth and eighth days. If parasites were present the line was defined as "resistant"; if absent, the line was termed "sensitive".

2.10 Genetic techniques

2.10.1 Conducting the cross and recovering the products of the cross.

The method adopted was based on the technique of Walliker *et al.* (1975 - modified by MacLeod (pers. com.). Fig. 2 illustrates the method used for a genetic cross between two phenotypically different lines of *P. chabaudi*. 10^6 blood forms of each line were

Figure 2. Method for genetic cross.



injected intraperitoneally into 5 mice each. Four to five days later a blood count and parasitaemia count were made for each group of mice. Equivalent numbers of parasites of each line were then mixed and injected intravenously in a splenectomized rat (1.0 ml inocula) in order to obtain an increase of gametocyte production (MacLeod and Brown, 1976). Splenectomy was carried out at least 24 hours before inoculation of parasites. Four to five days later, when a large number of gametocytes were present, the rat was exposed to mosquitoes. In these mosquitoes micro- and macro-gametes of each line are formed, and self and cross-fertilization occurs, within and between lines respectively. Mice infected from these mosquitoes thus contained parasites of recombinant as well as parental types. After mosquito transmission, stabilates of infected blood were stored in liquid nitrogen. Subsequent tests were carried out, using this stored material, on several different occasions.

2.10.2 Characterization of products of the cross.

(i) Testing to demonstrate the presence of recombinant forms among the products of a cross.

A standard drug test (page 24) was carried out on the uncloned products of the cross between chloroquine-sensitive and resistant lines. On the ninth day after inoculation all the infected mice were sacrificed and their blood analysed for enzyme type.

The object of this test was to determine whether the enzyme types originally associated with the chloroquine-sensitive parental parasite line were present among the products of the cross surviving the drug treatment.

Figure 3. Competition studies: Analysis of a blood form induced infection. Proportion of 50%: 50% resistant to sensitive parasites.

	DAY 0	DAY 3	DAY 10	2nd Peak
Resistant line (R)	(1)	(2)	(2)	(2)
Mixture (M)	(1)	(2)	(2)	(2)
Sensitive line (S)	(1)	(2)	(2)	(2)

(1) Inoculation of 10^6 parasites

(2) Mass tests and establishment of clones

(ii) Cloning and characterization of the cloned products of a cross

Cloning was carried out by dilution, using the method described previously (page 20).

The clones derived from the products of the cross and established on several occasions were analysed with respect to each parental character for full analysis of the progeny.

(iii) Control studies

Parasites of each parental line were treated similarly to the mixture (Fig. 2) undergoing simultaneous mosquito transmission into mice. These mice were used as donors for further passages, cloning and drug treatment as controls.

2.11 Competition studies

Competition studies between line 411AS (resistant to chloroquine) and line 289AS (the chloroquine-sensitive parental line from which the line 411AS had derived), were carried out in the absence of drug pressure, using both blood-induced and sporozoite-induced infections. Both lines were clones and were transmitted through mosquitoes into mice, at the beginning of the experiment.

2.11.1 Blood form-induced infections

(a) Experiment 1 (Fig. 3)

1×10^6 blood forms containing a mixture of 50% resistant : 50% sensitive parasites were injected intravenously into three mice. The parasitaemia in these mice was recorded every 48 - 72 hours for 35 days. On day 3, day 10 (after the first peak of infection) and during the 2nd peak of infection (around day 30), the parasites infecting one of these mice were tested for resistance. For these

Figure 4. Competition studies: Analysis of a blood form induced infection. Proportion of 90% : 10% (line M_2) and 10% : 90% (line M_3) resistant to sensitive parasites.

	DAY 0	DAY 3	2nd Peak
Resistant line (R)	(1)	(2)	(3)
Mixture M_1	(1)	(2)	(3)
Mixture M_2	(1)	(2)	(3)
Mixture M_3	(1)	(2)	(3)
Sensitive line (S)	(1)	(2)	(3)

(1) Inoculation of 10^6 parasites

(2) Mass tests

(3) Mass tests and establishment of clones.

tests (termed here "mass tests") blood forms of the mixture were injected into a mouse which was used as a donor for a drug resistance test (except for day 3, in which no intermediate donor mouse was used). For a mass test, a group of 7 mice were inoculated with 10^6 blood forms, five of which were treated with 3 mg of chloroquine for 6 days, starting on day of inoculation, the remaining two mice being undrugged controls (See Section 2.9.3.). Blood smears for parasitaemia counts were taken on day 6 and day 8.

Clones were established from the mixed infection on the same days as the mass tests described above. On each occasion blood forms of the mixture were injected into a mouse from which clones were derived on the 3rd day of infection (an intermediate donor mouse was not used for day 3 experiments). The standard resistance test was applied to each clone (page 24).

(b) Experiment 2 (Fig. 4)

In this experiment, 10^6 blood forms were mixed in different proportions of resistant to sensitive parasites. Line M_1 was a mixture of 90% : 10% resistant to sensitive parasites, line M_2 a mixture of 50% : 50% resistant to sensitive forms and line M_3 a mixture of 10% : 90% resistant to sensitive parasites. For each line groups of three mice were injected intravenously with blood forms of a mosquito transmitted infection.

On day 3 and during the 2nd peak of infection blood forms of each mixture were injected into an intermediate mouse which was used as a donor for mass tests as well as cloning, carried out as described in Experiment 1, above.

Figure 5. Competition studies: Analysis of a sporozoite induced infection. Proportion of 50% : 50% resistant to sensitive forms.

	DAY 0	DAY 12	2nd Peak
Resistant line (R)	(1)	(2)	(3)
Mixture (M)	(1)	(2)	(3)
Mixture (MM)	(1)	(2)	(3)
Sensitive line (S)	(1)	(2)	(3)

(1) Sporozoite infection of mouse

(2) Mass tests

(3) Mass tests and establishment of clones.

Figure 6. Competition studies: Analysis of a sporozoite induced infection from a blood-mixture of 90% : 10% (line M_1), 50% : 50% (line M_2) and 10% : 90% (line M_3) resistant to sensitive parasites.

	DAY 0	DAY 8
Resistant line (R)	(1)	(2)
Mixture M_1	(1)	(2)
Mixture M_2	(1)	(2)
Mixture M_3	(1)	(2)
Sensitive (S)	(1)	(2)

(1) Sporozoite infection of mouse

(2) Mass tests and establishment of clones.

Control infections of the parental lines alone, were examined, cloned and tested simultaneously.

2.11.2 Sporozoite-induced infections

Two experiments were carried out using sporozoites as a source for infection of mice.

(a) Experiment 1 (Fig. 5)

M line was derived from sporozoites from mosquitoes which had been permitted to feed on a blood infection of 50% : 50% resistant : sensitive forms which had undergone gametocyte differentiation. MM line was obtained by mixing mosquitoes of control lines in equal proportions, according to the number of oocysts which had developed per gut, per parental line. In this experiment therefore, the mixture of parasites occured only at the sporozoite stage.

(b) Experiment 2 (Fig. 6)

Further experiments were devised in which mosquitoes were infected by feeding on mice which had been injected with the 90% : 10%, 50% : 50% and the 10% : 90% proportions of resistant to sensitive parasites.

Observations on the course of these infections were made only at day 8 after transmission of infection through mosquitoes, for all lines R, M_1, M_2, M_3 and S, and at day 12 and day 35 (2nd peak of infection) for lines R, M and S of the first experiment. Line MM was tested at day 12 and day 60 (2nd peak of infection).

3. Results

SECTION I

Selection of P. chabaudi lines resistant to chloroquine

I.3.1 Attempts to produce chloroquine-resistance in line AS.

A chloroquine resistant line was derived from line AS using the continuous low drug pressure method. Table 1 illustrates the development of this line from the start of drug treatment (passage 1) until resistance was established (passage 6). The full history of the production of a resistant line is given in Diagram I.3.1 in Appendix. For the first two passages a drug dose of 2 mg/kg of chloroquine was administered for 5 days. On passage 3 (mice 314AS in Diag. I.3.1 in Appendix) an increase to 3 mg of chloroquine for 5 days eliminated all parasites. The blood of these mice was examined until day 10 when four mice out of five exhibited a relapse infection. These parasites were injected into further mice (327AS) and submitted to 3 mg of chloroquine for five days, for the next two blood passages 4 and 5 (327AS and 333AS). In these mice the sensitivity to drug treatment was significantly lower than that of the sensitive parent line tested under identical conditions.

Although in mice 333AS growth rate was low (R = 2.4), parasites in subsequent mice, passage 6 (356AS), exhibited a higher level of resistance. The infection was then transmitted through mosquitoes, drug pressure being maintained in mice 356AS until day 11 when mosquitoes were permitted to feed.

Parasites transmitted through mosquitoes into mice (390AS) and subsequently tested for resistance using the standard resistance test showed a lack of response to this treatment (411AS), while the sensitive parent line was eradicated when tested simultaneously.

Table 1. Production of a P. chabaudi, AS line, resistant to chloroquine

Passage Number	Drug dose (mg/kg)	Length of Treatment (days)	Resistance selected (1)	Control Sensitive (1)
1	2	5	6.07	
2	2	5	28.40	
3	3	5	0.0	
4	3	5	15.2	0.6
5	3	5	2.4	0.5
6	3	continuous	28.05 (2)	0.0
7	Mosquitoes			
8	0			
10	3	6	21.82 (3)	0.0

(1) R ratio (page 24)

(2) Resistance established

(3) Resistance established through mosquitoes (mice 411AS)

From passage 10 to 17 (411AS to 514AS)(Diag. I.3.1) weekly blood passages were carried out and the dose of chloroquine administered was increased up to 6 mg. These passages were carried out as described above, except that on day 5 after inoculation (after 4 daily doses of 3 mg of chloroquine) a single dose of 6 mg of chloroquine was administered. The surviving parasites were now submitted to 3 mg of chloroquine again on day 6 before being passages into new mice, on day 7. During the 7 passages the level of resistance was raised to the desired level of 6 mg of chloroquine, but this increase in resistance proved unstable, when drug pressure was relaxed. However, the parasite line still maintained its resistance to 3 mg (R = 36.3 for mice number 514AS).

Control studies showed that this dose eliminated the/sensitive line AS parasites using the standard drug test. It can be seen in Table 1 and Diagram I.3.1 that in passages 4 and 5 there appeared to be a slight increase in resistance in the control sensitive lines but in these passages mice were not re-weighed during drug treatment. In all subsequent tests, when the drug dose was adjusted on day 4 to take account of any increase in mouse weight, parasites were eliminated.

P. chabaudi line 411AS was thus established as the resistant line for further studies.

I.3.1 (a) Cyclical transmission of chloroquine resistant lines.

Table 2 represents the cyclical transmissions carried out on several different occasions following the establishment of line 411AS. All the lines transmitted were maintained under a/continuous drug pressure of 3mg/kg daily until the day of exposure to mosquitoes. No further chloroquine pressure was used until the first passage after each sporozoite transmitted infection.

Table 2. Cyclical transmission of P. chabaudi AS, resistant to chloroquine, under drug pressure.

Exp. Number	D_1 (1)	Parasitaemia mean % of drugged mice on day 6.	R Ratio for Day 6	% Parasitaemia of mouse exposed to mosquitoes, Day 6.	Day of exposure to mosquitoes	1st test after mosquito transmission. Parasitaemia, mean % of drugged mice on Day 6	R Ratio for day 6	Other information
543 AS	2.7. 1974	3.8	22.0	3.2	D 13 D 13 D 13	1.97 5.0 3.06	8.9 17.1 10,0	sporozoites infected intravenously sporozoite induced infection (feed)
605 AS	11.10 1974	4.65	33.1	6.8	D 11	1.47	9.8	sporozoite induced infection (feed)
606 AS	11.10 1974	3.8	26.0	5	D 11	1.04	23.91	sporozoite induced infection (feed)
634 AS	17.10 1974	3.16	37,06	(2)	D 12	0.4	13.0	On D7 splenectomized mice were infected with parasite blood forms from all treated mice.
657 AS	11.12 1974	7.47	59.1	(2)	D 13	1.47	7,0	On D8
764 AS	21.5 1975	6,7	32.0	(2)	D 12	2.99	14.9	Sporozoite induced infection 15 days after mosquito feeding.
						0.63	2.74	" 17 days " "

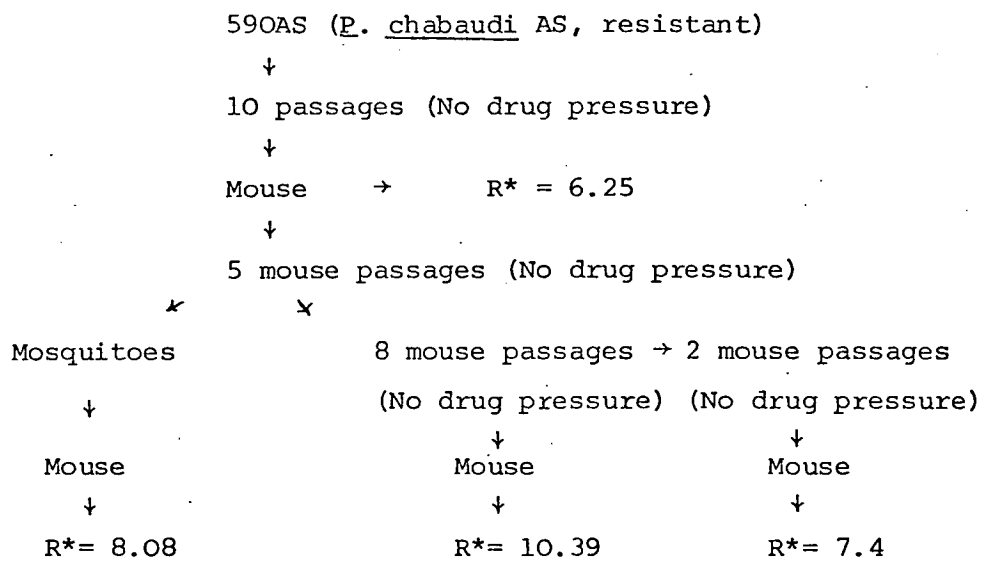
411 AS

(1) D_1 - day of inoculation and 1st drug dose

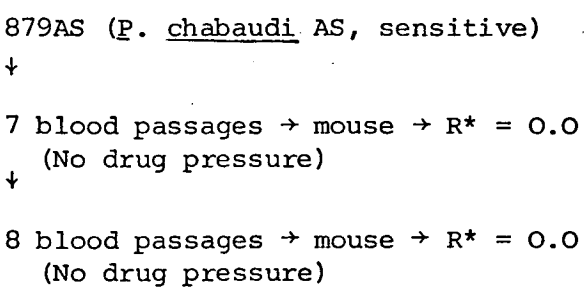
(2) Splenectomised rats used - parasitaemia not estimated.

Figure 7

(a) Stability studies on resistance.



(b) Control studies



* R ratio (see Section 2.9.2)

Each experiment showed that resistance to chloroquine had been maintained through the invertebrate phase of the life cycle. In experiment 543AS, one infected mouse was exposed to mosquitoes; two weeks later the sporozoite transmission was made either by intravenous injection of sporozoites prepared from dissected salivary glands or by allowing the infected mosquitoes to feed on a mouse. In experiment 634AS and 657AS, parasites were passaged into a splenectomized rat prior to mosquito transmission, to produce larger numbers of gametocytes. In experiment 764AS, two mice were separately exposed to infected mosquitoes, with a 48 hour interval between feeds. In all tests, as well as in results obtained in subsequent competition studies (see Results in Section III.3.2) chloroquine resistance was maintained through mosquitoes. This was necessary in the establishment of resistant lines for the purpose of a cross.

I.3.1 (b) Stability studies on chloroquine resistance.

To investigate whether parasites which survived chloroquine treatment possessed a stable resistance to the drug, studies on the stability of resistance were carried out by continuous blood passaging of resistant parasites in the absence of drug pressure.

Figure 7 represents the number of passages carried out and the results of subsequent tests for resistance. During 25 continuous blood passages ($\pm$ 170 generations), parasites were sub-inoculated into mice at passage 10, passage 23 and passage 25 and tested for resistance. At passage 15, parasites were transmitted through mosquitoes into mice, and then tested for resistance.

These studies were carried out for 25 weeks, the parasites being passaged on day 7 or day 8 after inoculation.

In order to study the influence of continuous blood passaging on resistance, in the absence of an invertebrate cycle, the parental sensitive P. chabaudi AS from which the resistant line had been selected was submitted to an identical routine and tested after 7 and 15 passages. Both tests confirmed sensitivity of the parental control and, thus, no effect of blood passaging on acquisition of resistance. For all tests carried out, resistance of line 411AS was thus proved stable and transmissible through mosquitoes in the absence of drug pressure.

I.3.2 Attempts to produce chloroquine resistance in line AJ.

The chloroquine resistant line 411AS was developed from a parent line which was resistant to pyrimethamine (page 22, Section 2.9.1). The purpose of this study was to produce also a line resistant to chloroquine, from a pyrimethamine-sensitive parent line. For this purpose, P. chabaudi AJ was used.

Two methods were used in these experiments:

(i) High pressure method:

Fifty mice infected with line AJ were treated with doses of 20 mg of chloroquine for four days. Fourteen days after the first drug dose, relapse parasites appeared in seven mice which were passaged and tested for resistance by treatment with 20 mg of chloroquine under identical conditions. No evidence of resistance to the drug was obtained as parasites were eradicated after the last drug dose. Studies were discontinued at this point.

Doses of 40 mg of chloroquine administered for four days to 10 infected mice eliminated the parasites for 12 days, after which 3 mice exhibited low parasitaemias (0.7%, 0.3% and 0.4%). These lines were passaged and tested, separately, using the same drug dose, which eliminated all the parasites, studies being discontinued at this point.

Doses of 60 mg of chloroquine administered to 10 infected mice eliminated all parasites and no relapses were detected for 28 days after the first drug dose.

(ii) Continuous low pressure method:

Full history of the results obtained is summarized in Table 3, and is given in Diagram I.3.2, in the Appendix.

Line AJ was first passaged through groups of mice (passages 1 and 2) in which drug doses of 3 mg/kg of chloroquine were administered for 4 and 3 days respectively. The dose was lowered to 1.5 mg (continuous exposure) for passage 3. Ten days later, parasites surviving this drug pressure were passaged into mice which were treated with 3 mg of chloroquine for 6 consecutive days without elimination of the parasites. The repetition of this treatment in passage 5, confirmed resistance of P. chabaudi AJ to this dose of chloroquine, (mice 524AJ). Clones were established from line 524AJ and drug tests confirmed resistance to chloroquine.

Table 3. Production of a P. chabaudi, AJ line, resistant to chloroquine.

Passage Number	Drug dose (mg/kg)	Length of Treatment (days)	Resistance selected (1)	Control sensitive (1)
1	3	4	0.0	-
2	3	3	1.72	0.0
3	1.5	continuous	29.8	-
4	3	6	4.8	0.0
5	3	6	7.76 (2)	0.0

(1) R ratios (page 24)

(2) Resistance established (mice 524 AJ)

I.3.2 (a) Cyclical transmission of chloroquine resistant lines.

Parasites derived from 524AJ and from clones established later were transmitted through mosquitoes and then tested for chloroquine resistance using the standard drug test. The two clones, resistant to the drug under serial blood passaging and drug pressure, were drug sensitive after mosquito transmission. However, when the parasite population from which these clones were derived had been cyclically transmitted resistance was still present after mosquito transmission.

These results suggested the existence of different types of resistance in the selected chloroquine resistant line 524AJ, the cloned lines established being unsuitable for a cross as chloroquine resistance was not transmissible through mosquitoes.

These results are shown in Table 4, and fully represented in Diagram I.3.2 (a), (b) and (c) in the Appendix.

I.3.3 In vitro culture results

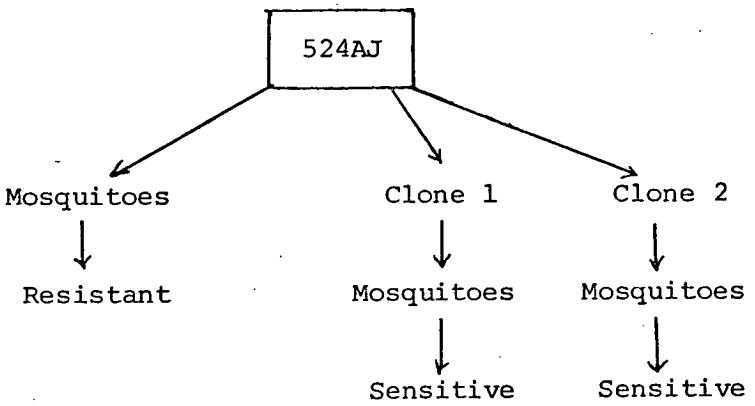
In vitro culture of P. chabaudi was carried out with lines 411AS (resistant to chloroquine), 290AS (the chloroquine sensitive parental line from which 411AS had derived) and 468AJ (a line sensitive to chloroquine). It was found that line 411AS developed schizonts and merozoites 1 - 2 hours earlier than the other lines. Reinvasion of uninfected red blood cells by merozoites was also detected, but as the culture would not last longer than 6 hours a continuous observation on the development of schizonts was not feasible.

Blood smears from in vivo controls confirmed earlier schizogony in the resistant line 411AS.

Inconclusive results were obtained on attempting to classify the levels of sensitivity of the different lines, due to technical difficulties which are discussed later (page 68).

Table 4 Cyclical transmission of 524AJ and 2 clones established from this resistant parasite population.

	Standard test[1]		Standard test[1]
524AJ	31.6	Mosquitoes	8.4[2]
clone 1	6.9	Mosquitoes	0.0[3]
clone 2	7.9	Mosquitoes	0.0[3]



(1) R ratios

(2) Maintenance of resistance through mosquitoes

(3) Loss of resistance through mosquitoes.

Figure 8. Phenotype of chloroquine resistant and sensitive lines, used in cross.

Lines	Phenotype
411AS	Resistant to 3 mg chloroquine (6 daily doses) Resistant to 15 mg pyrimethamine (4 daily doses) 6 PGD-2 LDH-3
96AJ	Sensitive to 3 mg chloroquine (6 daily doses) Sensitive to 15 mg pyrimethamine (4 daily doses) 6 PGD-3 LDH-2

Figure 9. History of cross.

Parental lines	
411AS	8 blood passages from 25/9/74 to 4/12/74 under drug pressure. → 657AS (R=59,1) → 663AS* → Mosquitoes → Mice 680AS (deep-frozen)
	283ASJ* → Mosquitoes Mice 329ASJ (deep frozen)
96AJ	8 blood passages from 25/9/74 to 4/12/74 → 707AJ (R=0.0) → 712AJ* → Mosquitoes → Mice 722AJ (deep frozen)

* Blood passage of parasites through splenectomized rats

** Deep frozen stabilates made.

SECTION II

Cross between 411AS and 96AJ

II.3.1 History of parental lines

After the production of the stable and transmissible chloroquine resistant line 411AS, genetic studies were carried out in order to investigate the genetic basis of the resistance produced and whether it showed recombination with other genetic markers. A cross was made between line 411AS and a line of different origin, 96AJ, which differed in 4 markers as shown in Figure 8.

II.3.1 (a) Analysis of products of cross.

In Figure 9 a summary of the genetic experiments carried out is shown. Deep-frozen stabilates were prepared of the products of the cross (mice 329ASJ) and the parental lines (mice 680AS and 722AJ).

(i) Tests for genetic recombination:

Capillaries taken from stabilates of each parental line and the products of the cross were removed from the deep-freeze and injected into separate groups of mice. 10^6 blood forms from each line were injected into three groups of mice, one group being undrugged, the second group submitted to treatment with 3 mg of chloroquine and the 3rd group submitted to 15 mg of pyrimethamine. Following drug treatment, parasites in each group were examined for enzyme-type (6 PGD and LDH). If no recombination had occured, drug-treatment in each group would be expected to eliminate all the drug-sensitive AJ type of parasites, only the drug-resistant AS parasite forms remaining (6 PGD-2 and LDH-3). Thus, if after drug treatment both forms of each enzyme were present in the surviving population, it could be concluded that new parasite forms had been produced by genetic recombination following the cross.

The untreated products of the cross would be expected to show all enzyme forms, but these might represent both parental forms as a simple mixed infection.

Table 5 represents the results obtained by drug testing the parasites derived from the cross between 411AS and 96AJ and shows that both enzyme forms of the parental sensitive line AJ are present after treatment of mice infected with the products of the cross. It could be concluded, therefore, that genetic recombination between drug-markers and enzyme-markers of the parental lines had occured. Similar results were obtained using stabilates from mice infected separately from the same group of mosquitoes.

(ii) Characters of clones derived from products of cross.

70 clones were established by dilution of blood forms of the products of the cross. Each clone was tested for enzyme-type and drug-response by injecting 10^6 parasites into three groups of mice each, as described in the previous section. Table 6 shows the characterization of the 70 clones obtained. Groups A - Q in Table 6 represent the 16 possible classes of character combinations. Groups A and Q represent line AJ- and AS-character classes respectively, and groups B - P are recombinant classes. It can be seen that despite the absence of clones in five groups (C, D, G, H and P), recombination occured between each of the parent-line markers.

32 of the seventy clones were chloroquine resistant, the R ratios for each of them being shown in Table II.3.1 in the Appendix.

II.3.2 Control studies

As controls, parasites of the 2 parental lines (mice 680AS and 722AJ) were cloned by dilution of infected blood after mosquito transmission of each line. Of 60 mice inoculated, for control 680AS, 24 clones were obtained and individually tested, the phenotype being for each clone, LDH-3, 6 PGD-2, pyrimethamine-resistant and chloroquine-resistant. The R ratios of each clone are given in Table II.3.2 in the Appendix.

Of 60 mice inoculated, for control 722AJ, 14 clones were obtained and individually tested, the phenotype being for each clone LDH-2, 6 PGD-3, pyrimethamine-sensitive and chloroquine-sensitive.

These results suggested, therefore, that mutation events were unlikely to have accounted for the recombinant types of parasites among the products of the cross.

Table 5. Results of drug tests of products of the cross and parent lines controls

Lines	680 AS			329 ASJ			722 AJ		
Drug-Test	UND*	3mg cq.(2)	15mg Pyr.(3)	UND	3mg cq.	15mg Pyr.	UND	3mg cq.	15mg Pyr.
(1)	10.4	0.35	0.25	12.2	0.47	0.08	11.9	(-)	(-)
LDH forms	3	3	3	2/3	2/3	2/3	2		
6 PGD forms	2	2	2	2/3	2/3	2/3	3		

(1) Figures represent mean parasitaemias (% of red blood cells infected) on day 6.

(2) cq = chloroquine

(3) Pyr. = Pyrimethamine

* UND. = undrugged.

Table 6. Characterization of 70 clones established from the products of the cross between 411AS and 96AJ.

Group	LDH Form	6PGD Form	Pyrimethamine Response	Chloroquine Response	Number of Clones
A	2	3	S	S	32
B	2	3	S	R	11
C	2	3	R	S	0
D	2	3	R	R	0
E	2	2	S	S	2
F	2	2	S	R	7
G	2	2	R	S	0
H	2	2	R	R	0
I	3	3	S	S	1
J	3	3	S	R	2
L	3	3	R	S	2
M	3	3	R	R	1
N	3	2	S	S	1
O	3	2	S	R	7
P	3	2	R	S	0
Q	3	2	R	R	4

Total number of clones: 70

R - resistant to drug pressure

S - sensitive to drug pressure

LDH-2 or -3 : electrophoretic forms of the enzyme lactate dehydrogenase

6PGD-2 or -3 : electrophoretic forms of the enzyme 6-phosphogluconate dehydrogenase.

II.3.3 Cyclical transmission and stability studies on a recombinant resistant clone.

It was shown in Section I.3.2 that cloned parasites resistant to chloroquine, derived from line AJ (pyrimethamine sensitive, LDH-2, 6 PGD-3) did not maintain their resistance after transmission through mosquitoes. From the cross carried out between lines 411AS and 96AJ, 11 clones were found in group B which possessed the enzyme forms characteristic of AJ, were sensitive to pyrimethamine and were resistant to chloroquine. Further studies on one of these clones (347ASJ) were carried out as follows:

(i) Cyclical transmission and tests for resistance of the mosquito transmitted parasites.

Line 347ASJ was tested for resistance before transmission through mosquitoes, using the standard drug-test. The R ratio for day 6 was 9.0. The line was then transmitted through mosquitoes into two mice. One mouse was infected with sporozoites from mosquitoes fifteen days after their "feed" on gametocyte carriers and the second with sporozoites after twenty days. After drug-testing the R ratios were R = 6.4 and R = 3.0, respectively.

(ii) Stability studies

347ASJ was continuously transmitted in the absence of drug pressure through mice for 19 weekly passages (made on day 7 or 8) and tests for resistance were carried out at passage 10 and passage 19, the R ratios being respectively R = 18.1 and R = 13.2. The resistance acquired by recombination during the cross was thus stable in the absence of the drug.

These results show therefore that, by genetic recombination, stable

chloroquine resistant lines were produced which possessed enzyme characteristics of line AJ and which were pyrimethamine sensitive. Previously, it had proved impossible to develop such lines by simple chloroquine selection on line AJ alone (Section I.3.2(a)).

III Competition studies

The object of these studies was to investigate the fate of chloroquine resistant mutants in mixed populations in the absence of drug-pressure. The stable chloroquine resistant line 411AS was mixed in different proportions with the sensitive line from which it had been derived by continuous low pressure. The following results were obtained:

III.3.1 Blood form-induced infections

(i) Results of experiment 1: 50% resistant: 50% sensitive blood forms

The mass tests and characteristics of clones established after 3 and 10 days and at the second peak of infection of this mixture are presented in Table 7. The second peak occured 30 days after inoculation of lines S and R, and 32 days after inoculation of line M, the peak of infection (3.4% red blood cells parasitized) being on day 35. At the start of the experiment each line was tested separately for chloroquine response the R ratios being R = 0.0 for the sensitive line and R = 12.27 for the resistant line. The results show that both sensitive and resistant forms are present at early stages in the infection, but that there was a predominant number of resistant parasites later, when relapse forms were tested. Thus, at day 3, 9 sensitive to 11 resistant clones were found, while at the second peak 0 sensitive to 9 resistant forms were found. From these results three conclusions were made:

1. The ratio of 9 resistant to 11 sensitive forms obtained on day 3 suggested that the proportion of 50 : 50 resistant to sensitive parasites had been correctly established.
2. An advantage of the chloroquine resistant forms is seen from the 9 : 0 resistant to sensitive clones obtained at the second peak of infection. At day 10, only two clones (both resistant) were established and because of the low number, no evidence for such advantage could be concluded.
3. Mass tests of line R, at the second peak, showed higher levels of R ratios comparatively to day 10.

(ii) Results of experiment 2: 90% resistant: 10% sensitive blood forms (M_1), 50% resistant: 50% sensitive blood forms (M_2) and 10% resistant: 90% sensitive blood forms (M_3).

The results of these studies are shown in Table 8.

Relapse infections (2nd peak) occured 30 days after inoculation for lines S and M_3 and R and 42 days after inoculation for lines M_1 and M_2. The parasitaemias at this stage were 0.41%, 0.16%, 1.07%, 0.08% and 0.5% for lines R, M_1, M_2, M_3 and S, respectively. The results obtained suggested, as in experiment 1(a) predominance of resistant parasites in the population mixtures as the infections proceeded. Clones were established only for 2nd peak infections. 8 resistant to 0 sensitive, 9 resistant to 0 sensitive and 4 resistant to 0 sensitive forms were obtained for lines M_1, M_2 and M_3 respectively.

At the second peaks, R values for mass-tests were higher than the values obtained for each line at day 3.

The values of R ratios for mass-tests obtained at day 3 did not vary accordingly to the proportions of resistant to sensitive parasites

inoculated (R = 8.30, 13.9 and 0.13 for lines M_1, M_2 and M_3 respectively).

Individual R ratios of resistant clones tested are given in Table III.3.1 in the Appendix.

III.3.2 Sporozoite-induced infections

Sporozoite-induced infections have the advantage over blood form-induced infections of resembling more closely the wild situation. Line M represents a cross between both lines as the mixture was made at the gametocyte stage of infection. Line MM represents a mixture carried out at the sporozoite stage, by mixing mosquitoes, infected separately, with sensitive and resistant parasites.

(i) Results of experiment 1: lines R, M, MM and S

The results of these studies are shown in Table 9.

At day 12 only mass-tests were carried out, the R ratios being R = 4.5, 2.26, 2.34 and 0.0 for lines R, M, MM and S respectively. Second peak infections occured after 35 days for lines R, M and S, and 60 days after for line MM. Clones established from each of these infections showed a preponderance of resistant forms (6 resistant to 2 sensitive forms for line M and 24 resistant to 0 sensitive forms for line MM) and an increase of R ratios obtained at the second peak of infection compared to day 12.

(ii) Results of experiment 2: lines M_1, M_2 and M_3

These results are shown in Table 10.

Mass tests were carried out for line R (resistant control) and the three different mixed populations at day 8 after sporozoite inoculation, the R ratios being R = 23.6, 16.6 and 4.98 for M_1, M_2 and

M_3 respectively. Transmission of line S (sensitive control) was unsuccessful. From the drug tests carried out with clones established for each line the following results were obtained: 0 sensitive to 11 resistant forms in line M_1, 0 sensitive to 8 resistant forms in line M_2 and 2 sensitive to 4 resistant forms in line M_3.

Individual R ratios of resistant clones tested are given in Table III.3.2 in the Appendix.

All results from the competition studies, carried out in the absence of drug pressure suggested an advantage of the resistant over the sensitive forms in both blood-form and sporozoite-induced infections.

Table 7. Competition studies: results from blood-form induced infection of a 50 : 50 proportion of resistant to sensitive parasites.

	DAY 3			DAY 10			2nd Peak		
Lines:	R	M	S(1)	R	M	S	R	M	S
Mass-test (R ratios)	-*	1.45	0	2.1	1.1	0	6.9	2.5	0
Number of clones tested	2	20	6	0	2	6	15	9	13
Number of:									
Resistants	2	11	0	0	2	0	15	9	0
Sensitives	0	9	6	0	0	6	0	0	13

* - Experiment not carried out.

(1) R - control resistant

M - Mixture of 50 : 50 resistant to sensitive forms.

S - Control sensitive.

Table 8. Competition studies: results from blood-form induced infection. 90:10, 50:50 and 10:90 resistant to sensitive parasites.

	DAY 3					2nd Peak				
Lines:	R	M_1	M_2	M_3	S(1)	R	M_1	M_2	M_3	S
Mass-test (R ratios)	9.7	8.3	13.9	0.13	0	31.7	25.9	21.6	18.1	0
Number of clones tested	-	-	-	-	-*	8	8	9	4	-*
Number of:										
Resistants						8	8	9	4	-
Sensitives						0	0	0	0	

* - Experiment not carried out.

(1) R - Control resistant

M_1 - 90% resistant : 10% sensitive form-mixture

M_2 - 50% resistant : 50% sensitive form-mixture

M_3 - 10% resistant : 90% sensitive form-mixture

S - Control sensitive.

Table 9, Competition studies: results from sporozoite induced infection, lines M and MM.

	DAY 12				2nd Peak			
Lines:	R	M	MM	S[1]	R	M	MM	S
Mass-test (R ratios)	4.5	2.26	2.34	0	10.9	24.3	15.6	0
Number of clones tested	-	-	-	-[2]	12	8	24	12
Number of:								
Resistants					12	6	24	0
Sensitives					0	2	0	12

(1) line M - mixed line obtained from a mixture of 50% : 50% resistant to sensitive blood-forms injected in a mouse and later exposed to mosquitoes.

line MM- mixed line, obtained from a mixture of 50% : 50% resistant to sensitive sporozoites.

(2) Experiment not carried out.

Table 10. Competition studies: results from sporozoite induced infection of lines M_1, M_2 and M_3.

Lines:	DAY 8				
	R	M_1	M_2	M_3	S[1]
Mass-tests (R ratios)	11.0	23.6	16.6	4.98	-[2]
Number of clones tested	-[3]	11	8	6	
Number of:					
Resistants		11	8	4	
Sensitives		0	0	2	

(1) - line M_1: 90% resistant : 10% sensitive form mixture.
line M_2: 50% resistant : 50% sensitive form mixture.
line M_3: 10% resistant : 90% sensitive form mixture.

(2) - unsuccessful transmission of line S (control sensitive) through mosquitoes.

(3) - Experiment not carried out.

4. Discussion

4.1 General considerations on mechanisms of drug-resistance

The appearance of parasites which survive drug treatment can be considered either as mutant forms in the population resistant to the drug which are selected by the drug, or as forms which, due to cell adaptations, temporarily resist the inhibitory effects of the drug under drug pressure. In malaria, resistance is of special importance when the new forms are able to transmit the character to their progeny, through mosquitoes. Any form of adaptation to the drug, which represents a reversible change, is of less importance as it does not mean a permanent alteration in the progeny.

Hinshelwood (1946), in a theory of direct induction, suggested that the contact of sensitive bacteria with a drug may shift the normal equilibrium of cellular chemical reactions to a new equilibrium which is less susceptible to the action of the drug. The proportion of cell components is altered and cell growth rate in the new conditions is dependent on a particular reaction path gradually selected. This theory, however, does not explain how changes of this type would be maintained in the absence of drug pressure, why clones from a resistant population may exhibit different degrees of resistance or how mutation to the drug can occur in the absence of the drug pressure. The mutation theories present two possibilities: either the parasite population exhibits variation in the response to drug treatment, due to random, spontaneous events unrelated to the environment of the organism, or there is a direct effect of the drug on the genetic material of the organism, by which mutations are induced and depending on their fitness, are maintained in the population. In the case of drug-resistance, highly

resistant lines due to mutation may not be produced after a single exposure to the drug, but by the gradual accumulation of several mutations, with an additive effect, over a number of generations. Cavalli-Sforza (1957) suggested that different mutations with presumably different fitness values would account for variance within a resistant population. Such variance was also explained by Yudkin's theory of clonal variation (Yudkin, 1953). The basis of the theory is that a bacterial cell, of whatever resistance, may give, on division, two cells with different and unequal resistance. Under drug pressure, cells from a cloned line would be selected, gradually, to the development of almost any level of drug resistance in the population. But, as Pollock (1960) pointed out, drug resistance can depend on the cumulative effects of a number of separate gene mutations; selection pressure at a given drug concentration would not necessarily operate in favour of a very highly resistant gene combination when a less highly resistant mutant would grow just as well. Also, the theory of clonal variation does not explain cases of high mutation rates when a high level of resistance is obtained very rapidly. Other reports on drug resistance were also difficult to explain satisfactorily using these models. For instance, in experiments carried out by Hinshelwood (cited by Demerec, 1957) on galactose fermentation in yeast, there were clear Mendelian phenomena, superimposed on which there were adaptive developments of the segregated characters themselves, the continuous levels obtained being suggested as dependent on both phenomena acting simultaneously.

The possible modes of origin of drug-resistance in micro-organisms are concisely summarized by Bryson and Szybalsky (1955), as follows:

(a) Resistance being dependent upon change of genotype, either by mutation which can be induced by the drug itself, by non-specific mutagenic agents or by genetic exchange phenomena such as sexual recombination.

(b) Resistance being dependent upon non-genetic change of phenotype, either by induction of a new physiological function such as an alternative pre-existing pathway, or a variety of phenotypic adaptations leading to resistance such as alterations in the permeability of the cell to the drug.

The development of resistance to chloroquine in the P. chabaudi lines described in the present work is discussed in relation to these theories in the following sections.

4.2 The nature of chloroquine resistance in P. chabaudi

4.2.1 Chloroquine resistance in line AS.

P. chabaudi (411AS) became resistant to chloroquine after continuous exposure to low levels of the drug. The resistance was maintained through mosquitoes and after continuous blood passaging in the absence of drug-pressure. This stability suggests that the resistance probably arose by mutation and selection in the presence of the drug. It was not possible to increase the level of resistance to higher than 3 mg/kg of chloroquine. This may be due to the fact that weekly passages of the treated parasites for only 7 passages did not allow such mutants to be detected. Several generations may elapse between the genetic event and its expression. However, parasites exhibiting an unstable resistance to higher drug levels were produced, suggesting adaptation to these levels. It is possible that mutations to higher levels could

have occured had the selection experiments continued for a longer period of time. Slower growth of parasites in the presence of drug pressure might increase the mutation rate.

The demonstration that line 411AS maintained its resistance after mosquito transmission has important implications concerning the spread of such resistant mutants in wild populations.

4.2.2 Chloroquine resistance in line AJ

Results obtained from low and high pressure selection will be discussed separately.

(a) Low pressure selection

The selection experiments using continuous low chloroquine pressure on line AJ produced evidence for the development of two forms of resistance to the drug, (i) a stable, heritable form, detectable after mosquito transmission and (ii) an unstable form, which could not be detected after transmission.

Table 4 (page 38) illustrates the results on which this evidence is based. After exposure to chloroquine, a parasite line denoted 524AJ was produced which exhibited resistance to the drug. When this parasite population was transmitted through mosquitoes, resistance could still be detected. Two clones established from 524AJ, before mosquito transmission, exhibited chloroquine-resistance, when drug pressure was maintained continuously. When the two clones were transmitted through mosquitoes, however, each proved sensitive to the drug.

The simplest explanation for these results is that during the chloroquine selection treatment of the parent sensitive line AJ, two different cellular alterations had occured, one being an adaptation to

the drug and the second a mutation event, conferring stable resistance. Line 524AJ may thus have comprised a mixed parasite population, some or all of the parasites being adapted and some (perhaps only a small number) containing the mutant gene. By cloning, only adapted forms were isolated, which lost their resistance after cyclical transmission. By transmitting the whole population 524AJ, adapted parasites were eliminated by subsequent drug treatment, but the mutant parasites were able to survive.

Further work will be necessary to establish whether this explanation is correct:

(i) A large number of clones could be isolated from line 524AJ and tested for drug response, and each clone then transmitted through mosquitoes in order to check the stability of resistance. This experiment would establish whether a mixture of mutants and adapted parasites was present in the resistant population, before mosquito transmission.

(ii) Continuous blood passaging of these clones in the absence of drug pressure would also distinguish mutants from adapted parasites which would be expected to lose resistance with passaging.

(iii) By cloning the resistant population obtained <u>after</u> mosquito transmission of 524AJ one would expect the vast majority of clones to be resistant, sensitive forms, if any, being due to rare events such as back-mutations, reversion to sensitivity or to other genetic processes such as supressors. These resistant forms would also be expected to maintain resistance through mosquitoes.

(b) High pressure selection

High doses of chloroquine were used and relapse parasites were tested with identical drug pressure. No resistant mutants were produced by these methods, but these experiments were incomplete. In further work using these methods, relapse parasites should be tested at lower drug doses, to which they might have become resistant. Also, in the work reported here, parasites which relapsed after the initial drug selection which were tested again using a similar course of treatment appeared to be sensitive. However, these experiments were discontinued immediately after drug treatment had finished; further examination of the second group of treated mice might have revealed resistant parasites later. Continuous passaging of relapse parasites for each line (20 mg and 40 mg) was not carried out for the eventual selection of a mutant line as in the method of Sautet et al. (1959) and Benazet (1965) successfully applied to P. berghei.

From these experiments it was concluded only that 60 mg of chloroquine administered for 4 days totally eradicated P. chabaudi infections of mice.

4.2.3 Relationship to pyrimethamine-resistance

(a) Line AS differed from line AJ in its response to another antimalarial drug, pyrimethamine, and in the enzyme forms of LDH and 6 PGD. The effect of such differences on the acquisition of resistance to chloroquine is unclear. Powers et al. (1969) in attempts to select a chloroquine resistant mutant in P. vinckei reported that success was obtained only by using a strain which was resistant to the maximum tolerated dose of pyrimethamine; no chloroquine-resistance developed when a pyrimethamine-sensitive strain was used. In human malaria it

seems clear that the occurence of chloroquine-resistance occurs independently of resistance to antifolate drugs. Thus, although chloroquine-resistant strains of P. falciparum occuring in South America were reported to be frequently resistant to pyrimethamine, chloroquine-resistant P. falciparum from Cambodia was usually sensitive to that drug (Peters, 1970), even though in both continents pyrimethamine was widely used for prophylaxis. In Africa, pyrimethamine was also widely used as a prophylactic and although resistance to that drug was reported, no knowledge of chloroquine resistant P. falciparum exists (Peters and Seaton, 1971). In the rodent malaria parasite, P. yoelii, isolates are innately and stably resistant to the drug dose of 40 mg of chloroquine/kg but the species is sensitive to pyrimethamine treatment (Warhurst and Killick-Kendrick, 1967). Similar results are reported with different P. berghei strains resistant to chloroquine (Schnitzer, 1966).

(b) The results of the genetic studies in the present work (to be discussed in more detail in Section 4.3) show clearly that separate genetic factors control resistance to chloroquine and to pyrimethamine. In the cross between lines 411AS and 96AJ, recombinant forms which were chloroquine-resistant but pyrimethamine-sensitive were produced.

Cyclical transmission of one of these clones (line 347ASJ) as well as continuous blood passaging in the absence of drug-pressure for 19 weeks confirmed the stability of the chloroquine resistance in this pyrimethamine sensitive line. These studies showed, therefore, that parasites resistant to chloroquine but sensitive to pyrimethamine can arise by genetic recombination and suggested their persistence in mixed populations, once produced.

It is not clear, therefore, why in the present work and in that of Powers et al. (1969), the production of stable chloroquine-resistance by drug pressure on pyrimethamine sensitive lines was less successful than when pyrimethamine-resistant lines were used.

4.3 Genetic Studies

In order to investigate the mechanism of transfer of chloroquine resistance among malaria parasites a cross between the chloroquine-resistant line 411AS and the sensitive line 96AJ was performed. An examination of the characteristics of 70 clones derived from the cross showed that chloroquine-resistance in P. chabaudi is a stable character which can undergo genetic recombination with other markers. Although limited by the number of clones analysed, no linkage with the 3 other markers used (pyrimethamine-resistance, LDH-3 and 6 PGD-2) was apparent. Each of the latter three markers also appeared to be unlinked, results which confirmed those of Walliker et al. (1975).

From a study of the numbers of clones in each of the 16 possible classes of progeny, the following observations could also be made:

(1) Although the total number of clones of parental type characters (36) was close to the predicted number (40), assuming random fertilization of gametes, there was a disproportionately high number of AJ type clones among these, 32 being of type AJ (group A) and only 4 of type AS (group Q). The significantly different number of parental type forms recovered from the products of the cross could be due to (a) AJ-type parasites outgrowing AS-type in splenectomized rats, (b) a disadvantage of pyrimethamine-resistant forms in splenectomized rats, and (c) sampling.

Clones were established from the progeny of the cross during early blood infections (day 3) in mice (in order to eliminate the possibility of establishing infections with red blood cells infected with more than one parasite); it seems unlikely, therefore, that selection against AS occured at this stage.

Because of the disadvantage of pyrimethamine-resistant parasites in competition with sensitive forms (Hall, 1976), due possibly to their dependence on high levels of dietary PABA (Hawking, 1953), hypotheses (a) and (b) may be the most probable cause of the disproportionate numbers. However, because of the relatively small number of clones isolated, it is also possible that sampling may have been a contributory factor.

(2) Despite the lack of AS characters, there were 32 chloroquine-resistant to 38 chloroquine-sensitive forms in the 70 clones analysed. The large number of chloroquine-resistant mutants detected (when compared to a deficiency of markers of the AS parental type line) could have been due to (a) an advantage of chloroquine resistant parasites, independent of the other genetic markers, (b) association of "chloroquine-resistance" and "infectivity" (e.g. larger number of merozoites being produced), (c) transmission of resistance by means other than a simple Mendelian fashion, with the spread of resistance-factors through the parasite population, (d) gene conversion processes, after recombination and (e) sampling. Of these different possibilities, sampling is the most probable as well as hypothesis (a) and (b) which will be discussed later.

Resistance to chloroquine due to extra-chromosomal, cytoplasmic mutations are, I believe, improbable. Any trophozoite is capable of producing male and female gametocytes so that any cytoplasmic character would be expected to be transmitted through mosquitoes. However, only reciprocal crosses involving the use of isolated microgametes could identify the true nature of cytoplasmic genetic markers.

(3) An absence of the expected recombinant type forms of groups C, D, G and H was observed. These groups coincide on possessing the enzyme-marker denoted LDH-2 and on being resistant to pyrimethamine, and it is possible that these forms could have been selected against at some stage of the cross.

(4) Results from control studies (Section II.3.2) did not suggest mutation events towards the production of chloroquine resistance in the parental line 96AJ or back-mutations towards sensitivity in the parental line 411AS, thus eliminating the possibility of these events in accounting for the recombinant types of parasites obtained in the cross.

4.3.1 Technical considerations and further research

Due to the significant difference in the number of parental type forms recovered from the products of the cross, an accurate genetic analysis on the frequency of recombination of the markers used was not possible.

Several limitations to these genetic studies exist due to the complex life cycle and requirements of malaria parasites. The most accurate analysis of a cross would be to carry out infections of

sporozoites of single mature oocysts which represent the product of a pure zygote. However, this technique is not yet practical. Crosses between the same lines should be carried out on several different occasions and the elimination of splenectomized rats as a source for higher number of gametocytes would be, possibly, advantageous. Establishment of clones, by micro-manipulation of blood forms would also eliminate sampling errors and allow a wider scope of material which could be obtained at different stages of the infection.

4.4 Competition Studies

4.4.1 Analysis of results

Analysis of the fate of chloroquine-resistant forms in mixed populations with sensitive parasites, was carried out in the final section of this work. While pyrimethamine resistant parasites were found to be at a disadvantage in competition with sensitive forms (Hall, 1976) chloroquine resistant mutants appeared to possess an advantage in both blood form induced infections and after mosquito transmission.

(a) Competition in blood form induced infections

(i) The results, based on mass tests for resistance and for analysis of clones, suggested a preponderance of resistant forms over sensitive forms, after the 1st peak of infection (day 7-8). Although only two clones were obtained at day 10 (see Table 7) a large excess of resistant forms were present at the 2nd peak analysis (9 resistant to 0 sensitive forms). The ratio of 9 sensitive to 11 resistant clones found at day 3 suggested that the initial mixed suspension

contained equal numbers of parasites of each line. Control studies carried out simultaneously (day 3 and 2nd peak) showed that mutation of the sensitive parasites to resistance was unlikely as the explanation for the ratios obtained.

(ii) When 3 different populations were studied (corresponding to 3 different proportions of mixed resistant and sensitive parasites, the ratios obtained at the 2nd peak were 0:8, 0:9 and 0:4 sensitive to resistant forms for mixtures M_1, M_2 and M_3 respectively, (see Table 8, page 49). Clones were not established at day 3 of these experiments, the R values for mass tests carried out at this stage being 8.25, 13.9 and 0.13 for lines M_1, M_2 and M_3 respectively. These figures could not be analysed statistically, mass tests being made only in order to obtain information on the chloroquine response of each mixed population, before clones were established. Higher R ratios were obtained at the 2nd peak of infection compared to day 3.

(b) Competition in sporozoite-induced infections

(i) Clones were established only at the 2nd peak of infection and mass tests were carried out at day 12 and at the 2nd peak. All the experiments showed similar results, indicating an apparent selective advantage of the resistant over the sensitive forms. From clones established at the 2nd peak, 24 were resistant and no sensitive forms were found (line MM), and a ratio of 6 resistant to 2 sensitive forms were obtained for line M. For the mass tests carried out 12 days after exposure to infected mosquitoes, similar

levels of resistance were obtained for line M and MM (R = 2.26 and 2.34 respectively) and similar but higher values were obtained at the 2nd peak tests (R = 24.3 and 15.6 respectively).

(ii) For lines M_1, M_2 and M_3 (See Table 10), analysed 8 days after exposure to infected mosquitoes, a ratio of 11:0, 8:0 and 4:2 resistant to sensitive clones was obtained. Mass tests carried out for each of these lines gave R ratios of 23.6, 16.6 and 4.98 respectively, thus suggesting still a preponderance of resistant forms.

4.4.2 Discussion of results and further research

(a) These results were unexpected in view of the fact that mutant organisms are normally selected against, in the absence of drug pressure. In the case of pyrimethamine resistant P. chabaudi, this appeared to be the case (Hall, 1976). Other studies on the behaviour of resistant forms when mixed with sensitive ones have been reviewed by Bishop (1958a) and Walker (1964). Studies by Cantrell (1956) with Trypanosoma (mixture of parasites was continuously passaged and tested) suggested elimination of the resistant parasites, in the absence of drug pressure. Studies in Bacteria have also suggested a disadvantage of resistant forms (Paine and Finland, 1948; Miller and Bohnhoff, 1950). Adoutte (1974) considered that most mutated mitochondria were overcome by wild-type mitochondria when mixed in the same Paramecium cells. For chloroquine resistance in P. chabaudi, however, the resistant form appeared to outgrow the sensitive in mixed infections.

A possible explanation for these results was suggested from in vitro culturing of these lines. These tests showed that earlier

schizogony occured in the chloroquine resistant population, 1 - 2 hours before the sensitive line, tested simultaneously. Blood smears from *in vivo* controls confirmed earlier schizogony in the resistant line 411AS and daily slides taken at the same time suggested that this phenomenon was periodic (24-hour cycle) and that a delay at some stage in the parasite growth *after* merozoite invasion occured to maintain such a periodicity.

After the 1st peak of infection (day 7-8), red blood cells would be scarce and would then, probably, be invaded by resistant merozoites, which were produced earlier. As gametocytes originate from merozoites and are produced mainly after such a peak, the resistant line would also be at an advantage in cyclical transmission.

(b) Another observation obtained from these studies was that relapse parasites of the parental resistant line and from the mixed populations exhibited a higher level of resistance (expressed as R) than those of day 3. The importance of these relapse parasites on the dissemination of chloroquine resistance is unknown as cyclical transmission, at this stage, was not carried out as these infections were of very short duration and low magnitude of infection. Soltys (1959) showed reduced sensitivity of an antigenically changed relapse strain of *T. brucei* towards suramin or antrycide, both *in vivo* and *in vitro* studies. Brown and Hills (1974) reported antigenic variation on *P. knowlesi* which was induced by antibodies. Whether host factors such as antibody production and its effect on the malaria parasites can induce changes in the parasite, which might have affected the drug response of these parasites is unknown. However, further experiments will be necessary to determine whether this observation is repeatable.

(c) Other than the suggestions already mentioned, more detailed competition studies should be carried out as follows:

1. for these experiments to produce results which are statistically testable the number of clones established, for each parasite mixture, should be increased by several fold.
2. oocysts obtained by dissection of mosquitoes infected with each separate line, resistant and sensitive, should be used as a source for the sporozoite-mixture studies, as oocyst counts in the present work were inconsistent and variable.
3. the earlier schizogony of the chloroquine-resistant line could be investigated in a cross in order to study its apparent linkage with chloroquine-resistance. Such a relationship would assume that during the selection of a chloroquine resistant mutant, either a different mutation had been simultaneously selected or that other effects were simultaneously being induced (pleiotropy).

4.5 Variation in results

From the evaluation of resistance (R values) during the course of this work, it can be seen that variation occured from one passage to another. Several factors may have accounted for this variation:

(a) one of the most important factors is the variability of response in experimental animals. McLaren and Michie (1956), Biggers et al. (1958) and Falconer (1960) found that mice of the C57 inbred strain - which was used in this research - were more variable in their response to the drug Nembutal than F_1 hybrids from crosses between two inbred lines. The rate at which a particular concentration of drug is attained in the

red cells and maintained, depends on the genotype of the host which controls the absorption of drugs from the alimentary canal (Vessel et al., 1971). Other than the importance given to the mouse strain chosen, other factors which may cause variation in drug response, include the sex and age of the host. Konopka et al. (1966) studied the effect of sex of host on protozoal chemotherapy and reported that female rodents infected with P. berghei were less protected by quinine than males, although opposite results were found with other drugs. Zuckerman and Yoeli (1954) found that male and female rats were equally susceptible to P. berghei infection and that females had more effective innate immunity than males. Ott (1969) suggested that the course of infection of P. chabaudi in mice was highly reproducible under various conditions including the use of young mice of approximately 20 g_m in weight. Ott (1969) and Wellde et al. (1966) suggested that intravenous injection of blood forms was more reliable than the intraperitoneal route. From all the information available and from the results obtained in this Unit it was decided to use young female C57 mice as hosts, which in preliminary work were found to exhibit longer infections when compared to males of the same age, and higher infections than in adults of either sex. Intravenous injection of inocula was not used as a general technique for inoculation of parasites, because of the time difference between the beginning of the inoculation procedure and its end, which would have complicated the timing of the first drug dose. Due to

these factors, the standard drug test for chloroquine resistance was carefully designed, treated mice being individually weighed on day 1 and day 4 so that drug dose remained constant.

(b) It can be seen from Table II.3.1 in the Appendix, that considerable variation in R ratios occured particularly among the progeny of the cross. Further work will be necessary to determine whether this was due to genetic recombination events (full resistance resulting from the cumulative effects of a series of mutations and not from a single step of considerable magnitude) or to host effects such as those described above.

(c) In attempts to eliminate the possible host-effects in the drug response, in vitro culturing of parasites was devised. Such technique, based on Rieckman's field test for P. falciparum (1968, 1971) was not equally successful with P. chabaudi due to the following reasons:

(i) a very large number of mice was necessary, in order to obtain a large volume of blood to distribute to the vials,

(ii) the dissection of mice had to be carried out aseptically in a short length of time,

(iii) individual parasitaemias and blood cell counts of the donor mice were not practical although necessary, as resistance was defined in terms of the number of trophozoites which had matured into schizonts, for each drug dosage and relatively to

undrugged controls. As no advantage was obtained, comparatively to the standard *in vivo* test for chloroquine resistance, *in vitro* culturing was discontinued.

5. General Conclusions

The objective of this project was to study the genetic basis of drug resistance in malaria. The project was limited to one rodent malaria model, *P. chabaudi*, and to one drug, chloroquine, pyrimethamine-resistance being used simply as a marker.

In one *P. chabaudi* line (411AS) resistance was produced which was stable in the absence of drug pressure and through mosquito transmission, the basis of which was investigated in a cross.

Although the level of resistance developed was low, the treatment of 3 mg/kg for 6 days eliminated sensitive forms and is similar to the recommended doses for suppression of human malaria (WHO, 1973). There was also evidence for the development of unstable resistance in line 524AJ which was lost after mosquito transmission, the basis of which is a subject for future research.

These results may have an important bearing on chloroquine-resistance in *P. falciparum*. Chloroquine-resistance has spread rapidly in this species in recent years, particularly in South East Asia. The work described here shows that stable resistant mutants can be produced after low level exposure to the drug and further, that such mutants appear to possess an advantage over sensitive forms even in the absence of the drug.

P. chabaudi may thus prove to be a more useful model system for

P. falciparum than the more widely used P. berghei, in which the only chloroquine resistant lines so far developed are unstable in the absence of the drug. Other possible advantages of using P. chabaudi instead of P. berghei for comparative studies with P. falciparum are: (a) it invades mature erythrocytes rather than reticulocytes, (b) it possesses a synchronous schizogony, (c) its gametocytes occur predominantly after the peak of infection and, (d) its sporozoites are more infective than those of P. berghei (Wery, 1967).

The fact that resistance to chloroquine, in the wild, has only been found with P. falciparum strains and not yet in Africa, suggests that innate parasite as well as host-mechanisms play an important role on the acquisition of resistance (Luzatto, 1974; immunity also plays an important role on the response to drug treatment since the parasites that survive the effects of the drug will be overcome by the immune mechanisms of the host (Bray, 1963; Luby et al., 1967). Reports by Hall and Canfield (1972) in studies comparing the response of black and white soldiers to infection with a resistant strain of P. falciparum from Vietnam have shown that the infection was less liable to develop as well as less difficult to eradicate in the black population group. Comparative studies on strains from different origins indicate that the basic chloroquine sensitivity of African strains is greater than the strains from South East Asia or South America (Peters, 1970). However, other than the possible appearance of spontaneous mutants, one should take into account the possibility of migrants, carriers of gametocytes of resistant P. falciparum into areas of known sensitivity, and eventual persistence due to recombination phenomena.

In a situation that can be denominated as a malaria threat, with the dissemination of resistant P. falciparum in South East Asia and

South America, the role of the geneticist is an informative one. The mechanisms of acquisition of resistance may not be completely understood but the fate of those resistant parasites in mixed populations, the stability in the absence of drug pressure and the pattern of cross resistance can be used in order to assist in the steps to take on the eradication campaigns against malaria.

It is hoped that the results from the studies here presented will provide useful information that will contribute to further progress in the field of chloroquine resistance.

6. Appendix

Diagram I.3.1 Production of a P. chabaudi, AS line, resistant to chloroquine

Date of inoculation and 1st drug dose (1974)	23.1	28.1	4.2	14.2	20.2	27.2	9.3	23.3	16.4	23.4	from 29.4 to 10.6	17.6	21.6	2.7
Mice Number	df.467(1) → 290AS → 296AS → 301AS → 314AS → 327AS → 333AS → 356AS → mosquitoes → 390AS → line deep-frozen (df.497)								→ 400AS → 411AS →		451AS 454AS 474AS 483AS 492AS 501AS 514AS	523AS →	clones established	→ 542AS → line deep-frozen (df.541)
Passage Number		1	2	3	4	5	6	7	8	9	10			
Drug Treatment	No CQ Pressure	2mg CQ 5 days		3mg CQ 5 days			3mg CQ continuous exposure	No CQ Pressure	No CQ Pressure	3mg CQ 6 days	3-6mg CQ 6 days	3mg CQ 6 days	No CQ Pressure	3mg CQ 6 days
Ratio R for Day 6.		6.07	28.40	0.0	15.2	2.4	28.05			21.82	For 514AS =36.3	31.3		55.2
Ratio R (Sensitive Control)					0.6	0.5	0.0			0.0	0.0	0.01		0.01

RESISTANCE ESTABLISHED

(1) - Stabilate df.467 contains parasite material established from a single sporozoite induced infection

CQ - Chloroquine

Diagram I.3.2. Production of a P. chabaudi, AJ line, resistant to chloroquine (CQ)

Date of inoculation and 1st drug dose :	7.5.74	20.5.74	29.5.74	3.6.74	13.6.74	17.6.74
Mice number :	379[(1)] → 468AJ → 476AJ → 495AJ → 500AJ → 518AJ → 524AJ → line deep-frozen (df 533)					
Drug Treatment	No drug Pressure	3 mg CQ 4 days	3 mg CQ 3 days	1.5mg CQ continuous exposure	3 mg CQ 6 days	3 mg CQ 6 days
Ratio R		0.0	1.72	29.8	4.8	7.76
Passage Number		1	2	3	4	5
Ratio R (sensitive control)			0.0		0.0	0.0

RESISTANCE ESTABLISHED

(1) stabilate df 379 contains cloned parasite material established from a mosquito transmitted infection.

Diagram I.3.2(a) Cyclical transmission of P. chabaudi AJ, resistant to chloroquine (Parasite population)

D_1*	3.10.74	10.10.74	18.10.74	30.10.74	7.11.74	19.11.74	3.12.74	10.12.74
Mice number	df.533 → 604AJ → 612AJ → 623AJ → 630AJ → 638AJ				→ 643AJ → Mosquitoes → 681AJ → 693AJ → 644AJ → Mosquitoes → 684AJ → 695AJ			
Drug Treatment	No CQ Pressure	3mg CQ 6 days	3mg CQ 6 days	3mg CQ continuous exposure	3mg CQ 6 days	3mg CQ continuous exposure	No CQ Pressure	No CQ Pressure 3mg CQ 6 days
R ratio		5.49	3.07	1.47		31.6 30.8		6.8 8.4

TRANSMISSION OF RESISTANCE CONFIRMED

REPEAT:

D_1*	22.875	5.9.75	20.9.75 22.9.75	29.9.75
Mice Number	df. 533 → 983AJ → 986AJ → Mosquitoes		→ 997AJ → 1002AJ → 998AJ → 1003AJ	
Drug Treatment	No CQ Pressure	3mg CQ Continuous exposure	No CQ Pressure	No CQ Pressure 3mg CQ 6 days
R ratio	2.4			7.1 27.2

TRANSMISSION OF RESISTANCE CONFIRMED

* - Day of inoculation and 1st drug dose.

CQ - Chloroquine

Diagram I.3.2(b) Cyclical transmission of Clone 1.

D_1*	7.11.74	13.11.74			28.11.74	10.12.74	25.12.74	3.1.75
Mice Number	644AJ → 650AJ → clones established → Clone 1 673AJ → Mosquitoes → 714AJ → 720AJ → Clone 2 677AJ (See next scheme)							
Drug Treatment	3mg CQ 6 days	3mg CQ 6 days	No CQ pressure		3mg CQ continuous exposure	No CQ Pressure	No CQ Pressure	3mg CQ 6 days
R ratio	31.6	9.9			clone 1=6.9 clone 3=19.1			Clone 1=0.0
								LOSS OF RESISTANCE

Diagram I.3.2(c) Cyclical transmission of clone 2.

D_1*	28.11.74	4.12.74	11.12.74	16.12.74	29.12.74	12.1.75	21.1.75	7.3.76	21.3.76	30.3.76
Mice Number	677AJ → deep frozen (df.592) → 6 blood passages under drug pressure → Mosquitoes → 823AJ → 827AJ → 683AJ → 703AJ → 706AJ → Mosquitoes → 723AJ → 724AJ									
Drug Treatment	3mg CQ continuous exposure	3mg CQ 6 days	3mg CQ 6 days	3mg CQ 6 days	No CQ Pressure	No CQ Pressure	3mg CQ 6 days	No CQ Pressure	No CQ Pressure	3mg CQ 6 days
R ratio	19.1	12.8	41.15	7.9			0.0			0.0
							LOSS OF RESISTANCE			LOSS OF RESISTANCE

* - Date of inoculation and 1st drug dose.

CQ - Chloroquine

Table II.3.1 Distribution of clones resistant to chloroquine established from the products of the cross between 411AS and 96AJ (R ratios)

Group	B	D	F	H	J	M	O	Q	
Total	11	O	7	O	2	1	7	4	32 clones
	3.92		0.90		3.83	21.0	0.52	10.0	
	5.2		0.95		11.8		0.85	10.9	
	6.5		3.2				1.0	37.0	
	6.61		5.0				1.70	66.5	
	7.14		6.4				3.60		
	8.21		11.2				13.8		
	11.0		12.1				15.8		
	11.57								
	16.40								
	23.5								
	25.0								
$\bar{X}$ =	11.36		5.67		7.81	21.0	5.32	31.1	

Note: All clones exhibiting values of $R \leq 1.0$ were classified as resistant to treatment with chloroquine after recording increase of parasitaemia and regular growth rate on day 8.

Table II.3.2 Control studies: R ratios of clones established from parental control 411AS

	5.9
	6.4
	12.7
	12.8
	14.2
	14.8
	16.0
	21.6
	23.7
	24.0
	27.1
	27.8
	30.3
	35.06
	37.76
	38.13
	45.05
	45.1
	46.8
	52.13
	52.4
	57.9
	70.9
	81.13
$\bar{X}$ =	33.3
Total:	24 clones

Table III.3.1 Competition studies: R ratios of resistant forms established from blood infections

	DAY 3		DAY 10		2nd Peak		2nd Peak			
Lines	R	M	R	M	R	M	R	M_1	M_2	M_3
Number of clones tested	2	11	0	2	15	9	8	8	9	4
R ratios	1.05	1.20		1.1	5.94	8.5	12.2	40.0	29.7	14.3
	9.90	1.38		7.8	11.22	10.5	13.9	51.5	57.2	42.9
		1.40			12.50	14.16	18.7	67.2	57.8	44.5
		1.94			13.70	16.6	27.8	72.2	58.2	62.2
		4.40			14.10	19.3	50.6	73.9	62.0	
		6.45			14.80	20.45	51.0	91.8	67.5	
		6.90			19.10	22.8	52.6	95.0	69.5	
		7.75			19.80	33.3	55.3	109.0	80.0	
		11.80			22.10	84.7			98.0	
		16.90			22.50					
		19.90			22.80					
					23.90					
					35.80					
					39.70					
					46.20					
$\bar{X}$ =	5.47	7.27		4.45	21.6	25.5	35.2	75.0	64.4	40.9

Table III.3.2 Competition studies: R ratios of resistant forms established from sporozoite-induced infections.

	2nd PEAK			DAY 8			
Lines	R	M	MM	R	M_1	M_2	M_3
Number of clones tested	12	8	24	0	11	8	6
R ratios	11.7	3.70	4.19		6.0	5.4	13.1
	22.0	14.9	14.49		9.4	6.3	13.6
	23.7	19.5	14.8		9.6	12.6	34.4
	37.6	21.8	14.9		10.7	15.3	55.2
	60.2	31.87	19.1		10.8	39.5	
	63.3	40.5	21.52		12.7	41.2	
	64.3		21.73		15.4	42.8	
	67.4		26.2		15.9	44.4	
	73.3		28.3		16.6		
	83.0		31.3		37.5		
	87.2		32.8		38.1		
	87.2		35.1				
			35.2				
			36.3				
			41.6				
			46.6				
			50.0				
			50.8				
			58.8				
			60.0				
			62.4				
			63.8				
			64.3				
			67.5				
$\bar{x}$ =	56.7	27.5	37.5		16.6	25.9	29.0

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I wish to certify that apart from the assistance listed above, all the experiments recorded in this thesis were planned and carried out by myself.

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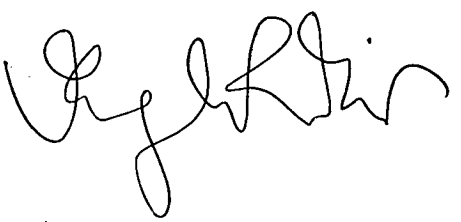

27th August 1976.

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Genetics of chloroquine resistance in malaria parasites

THE resistance of the malaria parasite of man, *Plasmodium falciparum*, to treatment with chloroquine is a growing problem, especially in South-east Asia and South America[1]. It is not known whether the emergence of resistance is attributable to the selection of resistant mutants under drug pressure, to the spread of naturally resistant forms in the parasite population or to adaptation to the drug by previously sensitive parasites. In malaria parasites of rodents, it has been shown that resistance to the antifolate drug pyrimethamine arises by mutation[2] and that the genetic factors involved can undergo recombination with other markers in crosses between resistant and sensitive parasite lines[3]. Resistance to chloroquine in rodent plasmodia seems to take several forms. *P. yoelii* isolates are innately resistant to the drug[4], whereas *P. berghei* and *P. vinckei* are sensitive. Stable chloroquine resistance has been produced in *P. vinckei* by drug selection in the laboratory[5] but resistance developed in this way in *P. berghei* is usually unstable in the absence of the drug[6].

I have investigated the genetic basis of chloroquine resistance in another rodent malaria species, *P. chabaudi*, which I believe serves as a useful model for *P. falciparum*. A chloroquine-resistant parasite line (411 AS) of *P. chabaudi* was obtained in mice by submitting blood forms of a sensitive line to a continuous low level drug pressure. Five mice (C57BL, 4–5 weeks old) were each injected with 10^6 blood forms and treated with oral doses of chloroquine at 2 mg kg^{-1} daily for five days from the day of inoculation. Mice were weighed individually at the start of treatment and on day four, the drug dose being adjusted if any change in weight had occurred. Parasites from that mouse which exhibited the highest parasitaemia were injected into a second group of mice and the treatment repeated. Five similar passages were made in mice, the dose being increased to 3 mg kg^{-1} from the third passage. Parasites which survived this course of treatment were then transmitted through mosquitoes into mice and tested for resistance. The line survived treatment of 3 mg kg^{-1} given for six days, whereas the drug-sensitive parent line, which had undergone a similar series of passages without drug pressure, was eradicated.

Table 1 Characterisation of 70 clones, produced by mixed infection of mosquitoes by lines 411AS and 96AJ

LDH form*	6PGD form†	Pyrimethamine response‡	Chloroquine response	Number of clones
2	3	S	S	32
2	3	S	R	11
2	3	R	S	0
2	3	R	R	0
2	2	S	S	2
2	2	S	R	7
2	2	R	S	0
2	2	R	R	0
3	3	S	S	1
3	3	S	R	2
3	3	R	S	2
3	3	R	R	1
3	2	S	S	1
3	2	S	R	7
3	2	R	S	0
3	2	R	R	4
Total				70

*LDH2 or 3, Electrophoretic forms of the enzyme lactate dehydrogenase.
†6PGD2 or 3, Electrophoretic forms of the enzyme 6-phosphogluconate dehydrogenase.
‡R, resistant to drug pressure; S, sensitive to drug pressure.

The stability of the resistance was tested by keeping the parasite line undrugged for 25 blood passages (6 months), tests for resistance being carried out at passages 10, 15 and 25. At passage 15, parasites were also transmitted through mosquitoes into mice and tested for resistance. In all tests (3 mg kg^{-1} for 6 d) the parasites were found to be resistant, the resistance thus proving stable in the absence of drug pressure.

The genetic basis of the chloroquine resistance was investigated in a cross between line 411AS and a sensitive line denoted 96AJ. The two lines differed additionally in three characters. Line 411AS was resistant to pyrimethamine (15 mg kg^{-1} administered for four consecutive days after inoculation of parasites), and possessed electrophoretic forms of the enzymes 6-phosphogluconate dehydrogenase (6PGD-2) and lactate dehydrogenase (LDH-3) respectively[7]. Line 96AJ was sensitive to both drugs and possessed enzyme forms 6PGD-3 and LDH-2.

The cross was performed using a technique described previously[3]. Equal volumes of parasitised red blood cells of each line were mixed and injected intravenously into a splenectomised rat, in order to obtain increased numbers of gametocytes. Five days later, mosquitoes (*Anopheles stephensi*) were permitted to feed on the mixed infection. At this stage micro- and macrogametes from each line were formed and, as a result, both cross and self fertilisation occurred. Fourteen days later, the infected mosquitoes were permitted to feed on mice. After mosquito transmission, the blood forms which developed were cloned by dilution, and each clone examined for drug response and enzyme type. As controls, the parent lines 411AS and 96AJ were transmitted through mosquitoes separately, and tested for drug response and enzyme type in a similar way. In tests for chloroquine resistance, 10^6 parasites of each clone were injected into two mice, which were treated with six daily doses of chloroquine at 3 mg kg^{-1}. Pyrimethamine resistance was tested similarly using doses of 15 mg kg^{-1} for 4 d. Electrophoretic forms of 6PGD and LDH were detected as in the method of Carter[7]. Seventy clones were derived from the products of the cross, the characteristics of which are shown in Table 1. Clones showing recombinant as well as parental type characters were present. The numbers of clones of the two parental types were significantly different, there being 32 of type 96AJ but only 4 of type 411AS. This suggests that selection against line 411AS had occurred, either when the parental lines were growing together in the rat before mosquito transmission or between zygote formation and the time at which clones were established. No significance, therefore, can be attached to the number of clones found in each recombinant class. It is of interest, however, that the proportion of chloroquine resistant to chloroquine sensitive clones found is 32:38, suggesting that the resistant form has no disadvantage compared to the sensitive form.

The results show that the chloroquine resistance which developed in *P. chabaudi* is a stable character, is inherited in simple Mendelian fashion, undergoes genetic recombination with other markers, and probably arose by mutation and selection in presence of the drug. Although the level of resistance developed is low, the treatment of 3 mg kg^{-1} for six days is sufficient to eliminate sensitive forms, and is similar to the recommended doses for suppression of human malaria[8].

The results, therefore, may provide an explanation of how chloroquine resistant forms could arise in the human malaria parasite, *Plasmodium falciparum*, in areas where the drug is widely used.

Further work is in progress in this laboratory to determine how the genetic factors involved can spread in populations of sensitive parasites. This knowledge is of particular importance

as chloroquine resistance in *P. falciparum* is widespread in regions such as South-east Asia, but not yet in Africa.

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