EFFECT OF COMBINATIONS OF CHLOROQUINE WITH SULPHADIAZINE OR PROGUANIL ON INDUCED CHLOROQUINE RESISTANCE IN *PLASMODIUM BERGHEI*

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INTRODUCTION

The rodent malaria parasite, **Plasmodium berghei** has been the most frequently used of all laboratory models for the study of malaria and antimalaria chemotheraphy. There are many strains of Plasmodium berghei of which the normal strain O or N-strain (Peters 1965) has been widely distributed for experiments in most laboratories. NS-strain is resistant to chloroquine but has the same morphlology as N-strain; **Plasmodium berghei yoelii 17x** has an inherent resistance to chloroquine and some other drugs but produces a less virulent infection than N-strain; N 67 **Plasmodium berghei** – like strain from Nigeria is innately chloroquine – resistant capable of invading normocytes as well as immature red cells and producing virulent rapidly lethal infection in mice. RC strain is resistant to both the 4 aminoquinolines and primaquine. Drug resistance by plasmodium species to antimalarial chemotheraphy has become a practical problem in many areas.

Drug resistance was easily produced to preguanil in the laboratory (Bishop 1959 and **P. falciparum** resistance to proguanil and pyrimethamine had been observed in the field (Archibald 1960, Dodge 1966, Lucas et al 1969, Fasan, 1970). Malaria parasites especially **P. falciparum** resistant to chloroquine have been encountered in South America and South-East Asia (Moore and Lanier 1961, Young and Moore 1961, Montgomery and Eyles 1963).

Drug combination and quinine have been found effective to varying extent in combating chloroquine – resistant **P. falciparum** and in preventing drug resistance in rodent malaria. (Ebiwawa, Namiki and Igarashi 1966, Bertelloni, Sheehy and Tigertt 1967, Hunter, Batey, Meleny and Sanders 1968, Peters, 1974).

In the study reported here the response of **Plasmodium berghei** made less sensitive to chloroquine was observed to combinations of chloroquine with either progunail or sulphadiazine.

METHODS

Swiss albino mice weighing 20–25Gm and randomly selected from a colony in the preclinical animal house, University of Ibadan, Nigeria were used. These were fed on rat and mice cubes made by Livestock Feeds Ltd., Ikeja, Nigeria. Groups of 5 mice were kept in each metal cage with wood shavings as bedding and in a room temperature of approximately 27°C. The strain of **Plasmodium berghei** used has been maintained in mice at the Department of Pharmacology, University of Ibadan for many years by blood transmission from mouse to mouse. It was originally brought to our department from the Department of Preventive and Social Medicine, University College Hospital by Dr. C. Adeniyi-Jones, and was maintained by Professor Dinah James till taken over by one of us. Chloroquine made by May & Baker Ltd. was obtained in injectable solution 40mg per ml.

Parasites were passaged from infected into clean mice when the donor mice had 30–40% parasitaemia because we have observed in our laboratory that the infection has been established and that parasites thus obtained were viable for intraperitoneal injection; reticulocyte infection had not become pronounced at this stage of infection. The inoculum injected contained approximately 1.6 x 107 parasitized red blood cells in 0.2ml sterile physiological saline.

Parasitaemia was evaluated by counting the number of parasitized cells per 100 red blood cells under a high power oil immersion light microscope on blood smear made on the 5th day of infection (taking day of infection as Day 0). Five to ten such fields were counted and the mean percentage parasitaemia calculated. Trophozoites, ring forms and schizonts could be seen on the same blood slide.

Both the therapeutic and prophylactic effect of drugs used were assessed as follows.

Therapeutic:

Each group of 10 mice was given a dose of chloroquine on Day 5, 6 and 7, the dose of drug being graded from one group to the other, a control group received only the solvent. Parasitaemia was assessed on Day 5 before drug injection and on Day 7, 9, 11 .... until the animals died or recovered, the therapeutic effect was estimated by the ability of a dose of the drug to clear the blood of parasites or to reduce the number of infected red blood cells.

Prophylactic:

Drugs were given in graded doses to different groups of 10 mice. The first of a graded dose of proguanil or sulphadiazine was given 30 minutes before parasite inoculation and the drug continued for 5 days.

Proguanil was given once daily while sulphadiazine twice daily. The prophylactic effect of a dose of a drug was assessed by the number of days that treated mice remained parasite free after inoculation compared with infected mice without drug treatment. The effectiveness was calculated as:

(Mean percentage Parasitaemia in infected animals without drug, on Day 5) minus (Mean percentage parasitaemia in infected animal treated with drug, post day 5).

Mean percentage Parasitaemia in infected animals without drug, on Day 5.

Inducing resistance or decreased sensitivity to Chloroquine –

The sub-curative dose of chloroquine was found from experiments described above as 30mg/kg. This dose was given in 0.2ml solution intraperitoneally daily to groups of mice on Day 5 and subsequently daily till day 12. Mortality rate was also recorded from Day 5 to assess the protection. Parasites were passaged from treated mice on Day 8 to new clean sets of mice which were also subsequently so treated. After 4 such passages it was established that the curative dose of chloroquine 40mg/kg failed to effect a cure in the mice as compared with parasites which had no previous contact with chloroquine.

Suppressive treatment of mice infected with chloroquine – resistance or Chloroquine – insensitive Plasmodium berghei

1. A control group was not treated during the 5th passage.
2. A second group was treated with 30mg/kg chloroquine as in previous passaging.
3. A third group was treated with 40mg/kg chloroquine on day 5, 6, 7.
4. A fourth group was treated with 40mg/kg chloroquine for three days and proguanil 5mg per kg for 5 days starting from day 5.
5. A fifth group was treated with 40mg/kg chloroquine for three days and sulphadiazine 50mg/kg twice daily for 5 days starting from Day 5.

Prophylactic treatment of mice infected with chloroquine resistant or chloroquine – insensitive P. berghei

1. Group I was not treated with any drug during the 5th passage.
2. Group II was treated as if undergoing the 5th passage with 30mg/kg chloroquine.
3. Group III was treated with 40mg/kg chloroquine for three days starting from Day 0 to Day 2.
4. Group IV was treated as for group III plus proguanil 5mg/kg for 5 days from Day 0 to 4.
5. Group V treated as for Group III, plus sulphadiazine 50mg/kg twice daily for 5 days from Day 0 to Day 4.

RESULTS

Chloroquine:

Graded doses of chloroquine, 10mg, 20mg, 30mg, 40mg, 50mg per kg body weight produced a graded **percentage effectiveness**, 40%, 50%, 60%, 99%, 100% respectively as calculated from the parasitaemia, the corresponding **percentage mortality** which was calculated for each dose from the total number of mice dead 3 days after the last dose of drug or 10 days after infection were 100%, 25%, 25%, 0%, 0%, while the **percentage protection** offered by each dose, was 0%, 75%, 100%, 100%, respectively, as calculated from the number of animals surviving for the same period specified above.

Proguanil:

Graded dose of proguanil given prophylactically for 5 days on Day 0 to Day 4 showed that the parasitaemia was related to the dose. 10mg/kg demonstrated no parasitaemia at the end of the 6th day of infection, higher doses caused death from drug toxicity, while smaller doses 9mg/kg and less allowed parasitaemia to develop by the 5th day after infection. The **percentage protection** calculated from the animal surviving up to the 9th day after infection were 0%, 50%, 100% respectively for the 3 doses, 4mg, 8mg, 10mg per kg body weight. The corresponding **percentage effectiveness** were 30%, 46% and 99%.

Sulphadiazine:

Only two doses of sulphadiazine were used; 100mg/kg did not allow of parasitaemia in mice at the end of 6th day of infection, while there was parasitaemia 5 days after infection with a dose of 50mg/kg. As calculated from mortality rate 9 days after infection, both 50mg and 100mg per kg offered 100% **protection** to the mice, however the **percentage effectiveness** calculated from the parasitaemia were 44% and 80% respectively.

Continuous Passaging in the presence of chloroquine:

The Mortality, Protection and Effectiveness during each passage in the presence of a suppressive dose of chloroquine 30mg/kg are shown in table 1, similar data in the presence of chloroquine 30mg/kg given prophylactically is shown in table 2.

Response of the passaged parasites to chloroquine or its combination with other drugs:

Suppressive treatment of mice given the 5th passaged parasites with chloroquine 40mg/kg, or chloroquine 40mg/kg combined with either proguanil 5mg/kg or sulphadiazine 50mg/kg, showed that the combinations produced higher percentage protection and effectiveness than chloroquine alone (Table 3).

The combinations offered 100% protection each compared with 75% for chloroquine alone; the combinations produced 84% and 76% **effectiveness** respectively for sulphadiazine and proguanil, while chloroquine alone had 50% **effectiveness.** Mice infected with parasites passaged free of previous contact with chloroquine 100% protection and 99% effectiveness with same dose of chloroquine 40m g/kg.

Prophylactic treatment of mice given 5th passage parasites with chloroquine 40mg/kg, chloroquine 40mg/kg plus sulphadiazine 50mg/kg and chloroquine 40mg/kg plus proguanil 5mg/kg gave the following results: the combination as well as chloroquine alone offered 100% **protection**; the **effectiveness** of **chloroquine sulphadiazine**, chloroquine-proguanil and chloroquine alone were 86%, 85% and 60% respectively (Table 3).

DISCUSSION

This study showed chloroquine 40mg/kg body weight to be a potent suppressive of rodent malaria in mice. A relapse occurred on the 9th day after infection when 3 doses of chloroquine were given on Days 5, 6 and 7, due to maturation of the parasites in reticulocytes. The parasites in reticulocytes had not matured during the treatment period and were unaffected by the drug which only attacked matured parasites in normocytes.

In vivo culture of **Plasmodium berghei** in a subtherapeutic dose of chloroquine 30mg/kg throughout the continuous passaging induced a degree of resistance to chloroquine, since sensitivity to the normal therapeutic dose (40mg/kg) decreased after the 5th passage.

Resistance of malaria parasites to proguanil had been observed both in the laboratory (Bishop and Birkett 1947, 1948) and in the field (Clyde and Shute 1959, Lucas et al 1969) while resistance to sulphadiazine was not common. Until the appearance of chloroquine resistant **Plasmodium falciparum** (Moore and Lanier 1961, Young and Moore 1961) the effect of drug combinations was not extensively investigated as was/is the practice with antibacterial and trypanocidal agents. Compared with chloroquine 40mg/kg alone, we found chloroquine-proguanil or chloroquine-sulphadiazine combination more potent in clearing the blood of the **Plasmodium berghei** which were passaged five times through mice exposed to 30mg/kg chloroquine. **Chloroquine-sulphadiazine** appeared to produce a higher percentage reduction of the parasitaemia than chloroquine-proguanil combination though both combinations offered the same percentage protection. Since neither proguanil nor sulphadiazine was found a good suppressive when used alone, and since the 5th passage parasites were less sensitive to the established curative dose of chloroquine 40mg/kg, the effectiveness of the combinations must have been due to potentiation of the effect of chloroquine 40mg/kg by the subtherapeutic dose of proguanil 50mg/kg or the subtherapeutic dose of sulphadiazine 50mg/kg.

There was no cross resistance between chloroquine and sulphadiazine or proguanil, presumably because the mechanism of action of chloroquine differs from those of the two drugs. Proguanil prevents parasite growth by interferring with the folic acid – folinic acid system in inhibiting the enzyme folic acid dehydrogenase and thus leading to inability to synthesize nucleic acids with consequent inpairment of parasites growth. Sulphadiazine competes with para-aminobenzonic acid which is essential for folic acid synthesis, this leads to formation of false folic acid which hinders parasites growth. Many mechanisms of action have been put forward to explain the antimalarial action of chloroquine though none seemed to have had the pride of place (Hahnetal 1966, Warhurst and Killick-Kendrick 1967, Macomber, Sprinz and Tousimis 1967, Peters 1970).

Attempts had been made to explain resistance to chloroquine by proposing several hypothesis (Macomber, O'Brien and Hahn 1966; Peters 1964, 1970 and 1971;

Homewood (and Warhurst 1970). However, resistance to chloroquine most probably depends on differences in capabilities of parasitised erythrocytes to permit the transport or accumulation of critical concentrations of the drug into the calls to effect cure. Macomber, O'Brien and Hahn (1966) found that chloroquine was concentrated in mouse erythrocyte parasitized by drug sensitive **Plasmodium berghei** 100 times that of the plasma, but only about 30 to 40% of this concentration was achieved in cells with drug – resistant parasites.

TABLE 1

Passage	% Mortality	% Protection	% Effectiveness
1st	25	75	60
2nd	75	25	22
3rd	25	75	41
4th	25	75	50
5th	75	25	20

Percentage Mortality and Protection offered to mice and the Percentage Effectiveness of the treatment on parasitaemia during continuous passaging of **Plasmodium berghei** through mice given suppressive treatment of chloroquine 30mg/kg for 3 days (Days 5, 6 and 7).

TABLE 2

Passage	% Mortality	% Protection	% Effectiveness
1st	25	75	60
2nd	75	25	25
3rd	25	75	40
4th	50	50	43
5th	25	75	42

Percentages Mortality and Protection offered to mice and the Percentage Effectiveness of the treatment on parasitaemia during continuous passaging of **Plasmodium berghei** through mice given prophylactic treatment of chloroquine 30mg/kg daily for 3 days (Days 0, 1 and 2).

TABLE 3

Effect of sulphadiazine-chloroquine and proguanil-chloroquine combinations on mice infected with 5th passaged chloroquine treated **Plasmodium berghei.**

Drug Combination	% Protection	% Effectiveness	Type of Treatment
Sulphadiazine 50mg/kg and Chloroquine 40mg/kg.	100	84	Suppressive
Proguanil 5mg/kg and Chloroquine 40mg/kg.	100	76	Suppressive
Chloroquine 40mg/kg alone.	75	50	Suppressive
Sulphadiazine 50mg/kg and Chloroquine 40mg/kg.	100	86	Prophylactic
Proguanil 5mg/kg. and Chloroquine 40mg/kg.	100	85	Prophylactic
Chloroquine 40mg/kg. alone.	100	60	Prophylactic

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