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Experimental Induction of Chloroquine Resistance in *Plasmodium berghei* NK65

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Abstract: The possibility of developing experimental chloroquine resistant *Plasmodium berghei* NK65 from chloroquine sensitive *Plasmodium berghei* NK65 was evaluated. Five mice of about 12 weeks old were inoculated with *Plasmodium berghei* (CQ sensitive strain). Exactly 72 h after inoculation and confirmation of parasitemia, these mice were treated with 10 mg kg⁻¹ body weight (b.wt.) every 48 h for one month. After this period, treatment was withdrawn for one week, following which sub-inoculation was made from each of the five mice to four new mice for each group respectively. Seventy two hours after parasitemia was confirmed in the sub-inoculated mice, two of the four mice in each group were treated with the correct dose of chloroquine, that is, 25 mg kg⁻¹ b.wt. daily for four days, while the rest were not treated. Parasitemia was monitored in all the groups for two weeks using thick and thin smears of blood films made from the tail vein of mice and stained with 10% Giemsa stain at pH 7.2. Two weeks after treatment with 25 mg kg⁻¹ b.wt. dose of chloroquine was stopped, four mice died in the first two groups, while one mouse each died in the remaining three groups. Six of the untreated mice from the replicate groups equally died beyond two weeks, while four survived. At death, the % parasitemia of mice that died were higher than those that survived after 2 weeks. These results suggest that those mice that survived two weeks after treatment with the right dose of chloroquine (25 mg kg⁻¹ b.wt. for 4 days) contained chloroquine sensitive *Plasmodium berghei* NK65 before they were cleared, while those that had persistence of parasitemia at relatively high level which resulted in their death contained chloroquine resistant *Plasmodium berghei* NK65. This finding should be of importance in studies involving development of new therapy for chloroquine resistant malaria.

Key words: *Plasmodium berghei* NK 65, chloroquine sensitive, chloroquine resistance

INTRODUCTION

Malaria is a mosquito borne disease closely tied to environmental conditions. It is one of the most prevalent diseases in the world and the leading parasitic cause of morbidity and mortality in the tropics (Martin *et al.*, 2004). Every year, thousands of cases are reported from all over Nigeria and the world in general (Martin and Hall, 2000).

About 2% of persons infected with falciparum malaria die, usually because of delayed treatment (Peter and Anatoli, 1998). The present global situation indicates a recent resurgence in the severity of the disease that malaria could still be described as one of the most important communicable diseases,

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with an annual incidence of 300-500 million clinically manifest cases and a death toll of 1-2 million people (Martin *et al.*, 2004; Miller *et al.*, 1994; More, 2002; David *et al.*, 2004). Mortality and morbidity due to malaria are matters of great concern throughout the world, especially in tropical and subtropical regions. The World Health Organization estimates that there are between 300 and 500 million new cases of malaria world wide, every year mostly in Africa, Asia, South Pacific Islands and South America, which causes, at least 3 million deaths (Alexandrous, 2007).

Chloroquine, a prominent antimalarial drug has been the first line drug used for the treatment of uncomplicated malaria over the last forty years. Chloroquine, though effective as a blood schizontocidal drug, is ineffective or only partially effective in resistant cases (Bickii *et al.*, 2000). Thus, effective chemotherapy has been hampered by the rise in the number of drug-resistant parasites. Currently the spread of chloroquine-resistant *Plasmodium falciparum* malaria is severely limiting our ability to treat malarial infection (Dawit *et al.*, 2006). Hence, severe and complicated cerebral malaria due to *Plasmodium falciparum* is further compounded by the chloroquine resistant parasites. Hence, the emergence and spreading of resistance to an increasing number of antimalarial drugs has been a major concern especially in Asia, Africa and South America (Silbley, 2001).

The emergence of multidrug-resistant *Plasmodium* is a world wide problem. Drug resistance is usually first recognize clinically as a recrudescence, when parasites reappear in the circulation after a period of latency following drug treatment. Recrudescence parasites, however, are not always resistant to the drug used for treatment (Ittarat *et al.*, 2003; Lemnge and Inambao, 1988; Schwartz *et al.*, 1983). Poor compliance and erratic absorption of drugs can lead to lower plasma concentrations and inadequate exposure to therapeutic concentration of drug (Slutsker *et al.*, 1990). Thus, despite adequate drug treatment, recrudescence may still occur (White, 1998).

Although major research efforts have been made to understand the mechanisms of drug resistance in malaria parasites, a definitive explanation remains elusive. *Plasmodium berghei* has been characterized as a useful model system for the study of drug resistance as aminoquinoline resistance in *Plasmodium falciparum* and *P. berghei* is similar in many biochemical and genetic aspects (Perez-Rosado *et al.*, 2002).

Many researches have been carried out with the aim of reducing the incidence of drug resistance in malaria and other parasites without much success. For the purpose of providing an effective weapon for evaluating new chemotherapeutic agents target at chloroquine resistant malaria, we attempt in the present study to assess the possibility of experimentally developing chloroquine-resistant *Plasmodium berghei* using chloroquine sensitive *Plasmodium berghei* NK65.

MATERIALS AND METHODS

Plasmodium berghei Parasite

The chloroquine sensitive *Plasmodium berghei* NK65 used for this study was obtained from Biochemistry Department, Nigerian Institute of Medical Research, Yaba, Lagos, Nigeria. The parasite was maintained by sub-passaging into healthy mice on a weekly basis throughout the duration of the study. Although *P. berghei* is generally used in rodent model for malaria, mice model was used in this study because of the high susceptibility of mice to *P. berghei* infection compared to laboratory rats and hamsters which are less susceptible (Kellick-Kendrick, 1978). The susceptibility of mice to *P. berghei* infection is equally supported by the study conducted by Pavia (1983).

The infection of the recipient mice was initiated by needle passage of the parasite preparation from the donor to healthy test animals via an intraperitoneal route as described by David *et al.* (2004) and Peter and Anatoli (1998). Briefly, *P. berghei* infected red blood cells obtained from the tail vein of infected mice was diluted with Phosphate Buffered Saline (PBS) so that each 0.2 mL that was subsequently injected contained approximately 10^6 - 10^7 infected red cells (parasite) per kilogram of body weight.

Induction of Chloroquine Resistance in *Plasmodium berghei* NK65

Five mice of about 12 weeks old were inoculated with *Plasmodium berghei* NK65 (Chloroquine sensitive strain) as described earlier. Parasitemia was confirmed in the mice after 72 h of inoculation. The five infected mice were treated with under dose of chloroquine (10 mg kg⁻¹ b.wt.) every 48 h for a period of one month. Thereafter, treatment was withdrawn for one week and then, sub-inoculation was made from each of the five presumptively chloroquine-resistant mice into four new mice for each group to produce five replicates consisting of a total of 20 mice.

Seventy two hours after parasitemia was confirmed in the presumptively chloroquine-resistant sub-inoculated mice, two of the four mice in each group were treated with correct chloroquine dose of 25 mg kg⁻¹ b.wt. for four days, while the rest two from each replicate were left untreated. A treated chloroquine-sensitive and an untreated chloroquine-sensitive group were also included as absolute control to test the actual efficacy of the chloroquine treatment

Estimation of Parasitemia and Statistical Analysis

Parasitemia was monitored in all the groups for two weeks starting from day 1 using thick and thin smears of blood films made from tail vein of mice (David *et al.*, 2004). The smears were stained with 10% Giemsa at pH 7.2 for 15 min and examined under the microscope to assess level of parasitemia.

$$\text{The percentage parasitemia was calculate as} = \frac{\text{No. of parasite in treated}}{\text{No. of parasite in control}} \times 100$$

Student t-test at p = 0.05 was used to compare the means for average parasitemia on day 14th after commencement of treatment and also percentage (%) casualty within two weeks for the various groups.

Evaluation of Stability to Chloroquine Resistance in *Plasmodium berghei* NK65

To establish the stability of chloroquine resistance in the presumptively chloroquine-resistant *Plasmodium berghei* NK65 strain, mice in the treated presumptive chloroquine-resistant group that died within 7 days despite treatment with chloroquine at 25 mg kg⁻¹ b.wt. for four days were considered to be highly resistant to chloroquine and were thus enlisted for evaluation of stability to chloroquine resistance. Sub-inoculation made earlier before the death of these mice and which were maintained for three subsequent generations (sub-passages) were used to inoculate 20 mice that were used in this study. The presumptive chloroquine-resistant sub-inoculated mice were divided into two groups of ten (10) mice each, with one group treated with chloroquine at 25 mg kg⁻¹ b.wt. for four days and the other group left untreated. A treated chloroquine-sensitive and an untreated chloroquine-sensitive group consisting of the same number of mice were also included as absolute controls to test the actual efficacy of the chloroquine treatment. Parasitemia was monitored in all groups for 14 days. Like in the earlier experiment, Student t-test at p = 0.05 was used to compare the daily means for average parasitemia in the various groups for 14 days following commencement of treatment.

This study was conducted at the Department of Biochemistry, Ahmadu Bello University, Zaria, Nigeria, between January and April, 2007.

RESULTS

To test for resistance, one week after resistance-induction treatment of 10 mg kg⁻¹ b.wt. every 48 h was withdrawn, sub-inoculation was made from five presumptively chloroquine-resistant sub-inoculated mice into four new mice each to produce five replicates of 20 mice. Upon establishment

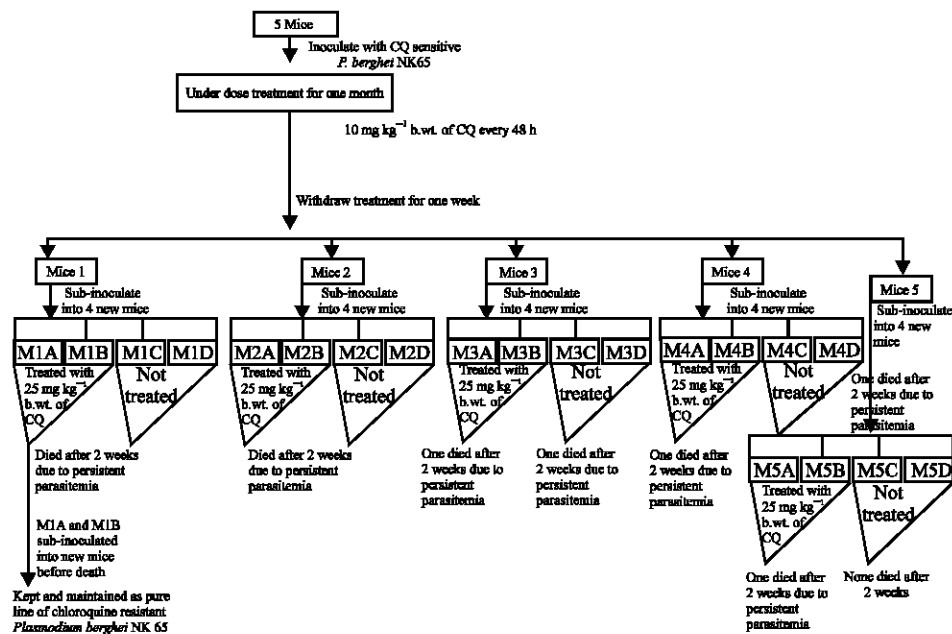


Fig. 1: Diagramatic presentation of the induction process of chloroquine resistant *Plasmodium berghei* NK65

of parasitemia after 72 h, two of the four mice in each replicate were treated with chloroquine at $25 \text{ mg kg}^{-1} \text{ b.wt.}$ for four days according to the design presented on Fig. 1 as follows: M1A and B, M2A and B, M3A and B, M4A and B, M5A and B and the other two in each of the replicates were left untreated as M1C and D, M2C and D, M3C and D, M4C and D, M5C and D.

Within two weeks of the treatment of the presumptively chloroquine-resistant sub-inoculated mice group with the correct chloroquine dose of $25 \text{ mg kg}^{-1} \text{ b.wt.}$ for 4 days, four mice died in the first two replicates, while one mouse each died in the remaining three replicates. Six of the untreated mice equally died within two weeks, while 4 survived. Among the casualties were 4 untreated mice from the first two groups (Fig. 1). The results also show that the percentage parasitemia of those mice that died were persistently higher than those that survived up to two weeks (Table 1). The parasitemia on which percentages were based were recorded before any death occurred. As may be seen on Table 2. No statistically significant difference ($p > 0.05$) was observed between the percentage parasitemia in the treated presumptive chloroquine-resistant group (Treated CQ-R) and the untreated presumptive chloroquine-resistant sub-inoculated mice untreated (Untreated CQ-R), but a statistically significant difference ($p < 0.05$) was observed between % parasitemia of the treated chloroquine sensitive *P. berghei* infected animal treated (Treated CQ-S) and either presumptive chloroquine resistant *P. berghei* infected animal that were treated with $25 \text{ mg kg}^{-1} \text{ b.wt.}$ for four days (Treated CQ-R) or left untreated with chloroquine (Untreated CQ-R) during the 14 days monitoring period.

When the experiment was repeated to establish the stability of the chloroquine resistance, there was no statistically significant difference ($p > 0.05$) between the % parasitemia of treated and untreated animals (Table 2). In the other hand, there was statistically significant difference ($p < 0.05$) between the % parasitemia of the chloroquine sensitive *P. berghei* infected animal treated with chloroquine and chloroquine resistant *P. berghei* infected animal treated with chloroquine (Table 2). It is noteworthy however, that essentially, no statistically significant difference ($p > 0.05$) was observed between the

Table 1: Average percentage parasitemia in previously under-dosed mice following treatment with 25 mg kg⁻¹ b.wt. of chloroquine

	n	Average % parasitemia					
		Day 0	Day 1	Day 2	Day 3	Day 4	
Treated CQ-R (M1A and B-M5A and B) (25 mg kg ⁻¹ b.wt.)	10	3.20±0.53 ^a	3.32±0.60	3.44±0.65	3.57±0.70 ^a	3.66±0.83	
Untreated CQ-R (M1C and D-M5C and D)	10	3.03±0.58 ^a	3.14±0.63	3.21±0.69	3.36±0.74 ^a	3.69±0.77	
Treated CQ-S 25 mg kg ⁻¹ b.wt.	10	3.74±0.13 ^a	2.68±0.11	1.70±0.13	0.49±0.10 ^b	0.00±0.00	
Untreated CQ-S	10	3.74±0.13 ^a	4.06±0.13	4.37±0.12	4.77±0.15 ^{bc}	5.11±0.17	
	n	Average % parasitemia					
		Day 5	Day 6	Day 7	Day 8	Day 9	
Treated CQ-R (M1A and B-M5A and B) (25 mg kg ⁻¹ b.wt.)	10	3.71±0.95	3.87±0.99	3.92±1.07	4.18±1.14	4.33±1.18	
Untreated CQ-R (M1C and D-M5C and D)	10	3.95±0.78	4.17±0.80	4.42±0.83	4.66±0.90	4.90±0.93	
Treated CQ-S 25 mg kg ⁻¹ b.wt.	10	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	
Untreated CQ-S	10	5.44±0.16	5.74±0.17	5.97±0.18	6.35±0.16	6.64±0.16	
	n	Average % parasitemia					Casualty in 2 weeks (%)
		Day 10	Day 11	Day 12	Day 13	Day 14	
Treated CQ-R (M1A and B-M5A and B) (25 mg kg ⁻¹ b.wt.)	10	4.54±1.24	4.78±1.31	4.96±1.35	5.17±1.41	5.33±1.45 ^a	53.84
Untreated CQ-R (M1C and D-M5C and D)	10	5.11±0.95	5.36±0.99	5.64±1.03	5.87±1.06	6.11±1.11 ^a	46.15
Treated CQ-S 25 mg kg ⁻¹ b.wt.	10	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00 ^b	0.00
Untreated CQ-S	10	6.91±0.16	7.24±0.16	7.57±0.19	7.81±0.20	8.11±0.22 ^d	95.50

Values with different superscripts vertically and horizontally differ statistically ($p < 0.05$), M1A and B to M5A and B consist of 2 mice each from the five replicates that were treated, M1C and D to M5C and D consist of 2 mice each from the five replicates that were not treated; Treated CQ-R; Treated presumptive chloroquine resistant group with 25 mg kg⁻¹ b.wt., Untreated CQ-R; Untreated presumptive chloroquine resistant group), Treated CQ-S; Treated chloroquine sensitive group (25 mg kg⁻¹ b.wt.), Untreated CQ-S; Untreated chloroquine sensitive group

Table 2: Stability of chloroquine resistance in *Plasmodium berghei* after three generations

	n	Average % parasitemia							
		Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Treated CQ-R (25 mg kg ⁻¹ b.wt.)	10	4.12±0.11 ^{ef}	4.41±0.13	4.70±0.13	5.00±0.14 ^{ef}	5.38±0.14	5.72±0.15	6.07±0.15	6.48±0.14
Untreated CQ-R	10	4.03±0.10 ^{ef}	4.36±0.12	4.65±0.11	4.99±0.12 ^{ef}	5.37±0.11	5.73±0.10	6.12±0.09	6.39±0.12
Treated CQ-S 25 mg kg ⁻¹ b.wt. (treated)	10	3.74±0.13 ^{ef}	2.68±0.11	1.70±0.13	0.49±0.10 ^g	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Untreated CQ-S	10	3.74±0.13 ^{ef}	4.06±0.13	4.37±0.12	4.77±0.15 ^{kl}	5.11±0.17	5.44±0.16	5.74±0.17	5.97±0.18
	n	Average % parasitemia							
		Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14	
Treated CQ-R (25 mg kg ⁻¹ b.wt.)	10	6.89±0.16	7.20±0.16	7.49±0.15	7.83±0.15	8.11±0.14	8.51±0.12	8.89±0.15 ^{sh}	
Untreated CQ-R	10	6.78±0.10	7.16±0.08	7.50±0.09	7.88±0.08	8.16±0.09	8.53±0.08	8.91±0.09 ^{sh}	
Treated CQ-S 25 mg kg ⁻¹ b.wt. (treated)	10	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	
Untreated CQ-S	10	6.35±0.16	6.64±0.16	6.91±0.16	7.24±0.16	7.57±0.19	7.81±0.20	8.11±0.22 ^{sh}	

Values with different superscripts vertically and horizontally differ statistically ($p < 0.05$), Treated CQ-R; Treated presumptive chloroquine resistant group with 25 mg kg⁻¹ b.wt., Untreated CQ-R; Untreated presumptive chloroquine resistant group), Treated CQ-S; Treated chloroquine sensitive group (25 mg kg⁻¹ b.wt.), Untreated CQ-S; Untreated chloroquine sensitive group

percentage casualty in the Treated CQ-R and the Untreated CQ-S groups after 7 days of treatment (data not shown), although at two weeks post commencement of treatment a statistically significant difference ($p < 0.05$) was observed between the two groups (Table 2).

DISCUSSION

Those mice that survived beyond two weeks after treatment with the correct chloroquine dose of $25 \text{ mg kg}^{-1} \text{ b.wt.}$ contained chloroquine sensitive *Plasmodium berghei* because the parasitemia disappeared completely. On the other hand, those mice that died within 2 weeks after treatment with the right dose of chloroquine contained chloroquine resistant *Plasmodium berghei*. There was persistent parasitemia at a very high level in this group which led to their death. This chloroquine resistant parasite are likely to have been induced by the under dose chloroquine treatment ($10 \text{ mg kg}^{-1} \text{ b.wt.}$) of the infected animals before they were treated with the right dose of chloroquine i.e., $25 \text{ mg kg}^{-1} \text{ b.wt.}$ daily for 4 days.

It is not clear why a marginally higher but statistically insignificant differences was observed in the casualty levels in the Treated CQ-R and the Untreated CQ-R (Table 1), but it is known that even for untreated malaria infection, spontaneous recovery may occur, while animals under treatment may also die, because of a number of factors including, resistance and the immune status of the infected organism. The observation that no statistical difference ($p > 0.05$) was evidenced between the casualty in the Treated CQ-R and the Untreated CQ-S groups after 7 days of treatment suggests that chloroquine treatment had no effect on Treated CQ-R (presumptive chloroquine- resistant group treated with $25 \text{ mg kg}^{-1} \text{ b.wt.}$ daily for 4 days), indicating that indeed, resistance to chloroquine had developed in this organism. This drug resistance may be as a result of recrudescence.

In a similar study carried out by Nakazawa *et al.* (2002) to determine whether high doses of chloroquine could prevent recrudescence, mice were given different doses of chloroquine ranging from $10\text{-}60 \text{ mg kg}^{-1} \text{ b.wt. day}^{-1}$ for 3 days once the initial parasitemia had reached 10%. Parasites decreased to an undetectable level during treatment and reappeared 4-8 days after cessation of treatment. All the mice treated with chloroquine showed recrudescence. There was a tendency for time from cessation of treatment to 0.01% parasitemia to increase as dose of chloroquine was increased. The number of dormant parasites appeared to be constant, so that the occurrence of recrudescence did not differ between mice treated irrespective of the dose of chloroquine. When treatment was discontinued, dormant parasites were reactivated and began to multiply. These parasites were found to be chloroquine resistant (Nakazawa *et al.*, 2002).

Dieckmann and Jung (1986) showed by using highly synchronous cultures, that pyrimethamine inhibited schizont formation but did not affect segmentation of mature schizonts, merozoite invasion or development of ring stage parasites. Ring stages were not susceptible to d-sorbitol treatment. These studies demonstrated that short-term exposure of ring stage parasites did not eliminate the parasites. If ring stages were present for longer than the treatment period; these parasites must have survived drug treatment. They therefore proposed that dormant ring forms caused recrudescence (Nakazawa *et al.*, 1995, 2002).

Parasites at the merozoite stage are probably also metabolically quiescent because merozoites have a short half-life and attach and penetrate new erythrocytes quickly. If parasites remain as merozoites, they may also survive drug treatment. It is difficult to detect such latent merozoites in culture or in circulation, because they rapidly invade erythrocytes after schizont rupture (Shusuke, 2005). Other workers (Landau *et al.*, 1995, 1999) have demonstrated that merozoites could be found in lymphatic networks when a heavy infection was introduced into mice and proposed that latent merozoites escape drug treatments by residing in macrophages or in neutrophil leukocytes. They

therefore examined the possibility that merozoites in phagocytes can cause recrudescence in *Plasmodium berghei* NK65 infection. It is more likely that dormant parasites may cause recrudescence of the infection following treatment.

Recent evidence has linked glutathione (GSH) pools to drug resistance in *Plasmodia* marked increase in both GSH levels and GSH-related enzymes activity was reported in *P. berghei* and *P. falciparum* lines resistant to chloroquine as compared to sensitive ones. The observation that after three generations, Treated CQ-R (presumptive chloroquine resistant group treated with 25 mg kg⁻¹ b.wt.), Untreated CQ-R (untreated presumptive chloroquine resistant group) and Untreated CQ-S (untreated chloroquine sensitive group) showed no statistically significant difference ($p > 0.05$) average levels of parasitemia within 14 days of treatment, as opposed to the Treated CQ-S (chloroquine sensitive group treated with 25 mg kg⁻¹ b.wt. for 4 days) (Table 2), appears to strongly suggest that the newly developed chloroquine resistance had become established in the *Plasmodium berghei* NK65.

CONCLUSION

In this study, we have demonstrated that chloroquine resistant *Plasmodium berghei* NK65 can be developed in the laboratory from chloroquine sensitive *Plasmodium berghei* through under dose treatment with chloroquine. This finding will be of important application in studies involving development of effective therapy for chloroquine-resistant malaria.

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