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Human malaria in C57BL/6J mice: An *in vivo* model for chemotherapy studies

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The present work deals with the development of *Plasmodium falciparum* stages in mouse model and its potential for the study of efficacy of antimalarial drugs. C57BL/6J mice were infected with multidrug resistant *P. falciparum* strain then treated with arteether and artesunate. A response was observed to antimalarial drugs in terms of decrease in parasitemia. Mice infected with *P. falciparum* strain were successfully cured after treatment with either arteether or artesunate. The speed of parasite clearance time and burden of parasitemia differed for each drug and matched the previously reported observations, hence stressing the relevance of the model. These findings thus suggest that *P. falciparum.* infected human RBC (iRBC) – C57BL/6J mice can provide a valuable *in vivo* system and should be included in the short list of animals that can be used for the evaluation of *P. falciparum* responses to drugs.

Keywords: Arteether, Artesunate, Parasitemia, *Plasmodium falciparum*

For thousands of years malaria swept through the ranks of human kind like a scythe in the hands of an angry god. *Plasmodium falciparum* malaria alone is responsible for over 800,000 deaths a year, with many of these fatalities occurring in infants in Africa[1]. In the absence of a long awaited effective vaccine, antimalarial drugs remain the main means to control morbidity and mortality due to *P. falciparum* malaria.

There are several families of drugs used to treat malaria. Chloroquine (CQ) was introduced in the 1940s and for many decades served as a cheap and reliable drug. However, due to the increasing emergence of chloroquine–resistant strains of the malaria parasite it is no longer effective against *P. falciparum*[2,3]. Due to widespread reports of Chloroquine ineffectiveness against the malaria parasite in endemic countries outside the African continent, the Federal Government of Nigeria officially banned the use of Chloroquine in 2004. This policy was not affected until 2010. However, Chloroquine is still in circulation because it is still available over the counter in chemist shop. In the face of these challenges regarding chemotherapy of malaria, artemisinin and its derivatives (artesunate, arteether, artemether and dihydroartemisinin) have given renewed hope for combating resistant malaria[4,5].

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At present it is strictly controlled under WHO guidelines as it has proven to be effective against all forms of multi-drug resistant *P. falciparum.* It is also only given in combination with other anti-malarials. Arteether is an ethyl ether derivative of dihydroartemisinin. It is used in combination therapy for cases of uncomplicated resistant *P. falciparum.* The recommended dosage is 150 mg/kg per day for 3 days given by im injections. In rats, oral and intramuscular studies were carried out at three dose levels at 9, 17.5 and 30 mg kg^{-1} and im route of administration of the isomeric mixture may be more beneficial for malarial chemotherapy[6]. With the exception of a small number of cases demonstrating neurotoxicity following parenteral administration no side effects have been recorded.

Artesunate is a hemisuccinate derivative of the active metabolite dihydroartemisin. Currently it is the most frequently used of all the artemisinin-type drugs. Its only effect is mediated through a reduction in the gametocyte transmission. It is used in combination therapy and is effective in cases of uncomplicated *P. falciparum*. The dosage recommended by the WHO is a 5 or 7 day course (depending on the predicted adherence level) of 4 mg/kg for 3 days (usually given in combination with mefloquine) followed by 2 mg/kg for the remaining 2 or 4 days.

Hitherto, immunodeficient mice are been widely used as xenogeneic transplantation models for

in vivo investigations of human cells and organs. Badell *et al*.[7] developed a model in which *P. falciparum* – parasitized human red blood cells *(P. falciparum*-hu RBC) can be grafted into immunodeficient (bg/bg xid nu/nu) BXN laboratory mice (*P. falciparum*-hu RBC-BXN)[8]. The present study has been undertaken to develop C57BL/6J mice model receiving *P. falciparum* parasitized human red blood cells. The *P. falciparum* parasitemia can be obtained in these mice for several weeks. The response of the antimalarial drugs was evaluated by observation of parasitemia and parasite clearance time (PCT).

The aim of the present work is to investigate the potential value of this new animal model for antimalarial drug studies. This new mice model eventually provide a valuable tool with which to investigate the biology of this malaria parasite under *in vivo* conditions and therefore help the researchers in the fields of chemotherapy and vaccine development. Although many new antimalarial drugs are commercially available, the experimental *in vivo* model is not well developed for chemotherapeutic studies on human malarial pathogen *P. falciparum.* Hence, a new vista has been opened to develop C57BL/6J mice as an *in vivo* model.

Materials and Methods

Mice—The Jackson Laboratory genetic management system is the most widely used "genetic background" for genetically modified mice for use as models of human disease. They are the most widely used and best-selling mouse strain, due to the availability of congenic strains, easy breeding, and robustness. It is unusually sensitive to pain and to cold and analgesic medications are less effective in it[9].

Freshly bred 12-week old male C57BL/6J mice developed by C.C. Little in 1921 were used for the experimental studies. Mice were purchased from National Institute of Nutrition (NIN), Hyderabad and the experiments were conducted according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiment on Animals (CPCSEA). The mice were acclimatized to the laboratory conditions for one week and protected from mosquito bite by using mosquito mesh. The mice were maintained under normal room temperature in well spaced polyvinyl cages, fed with formulated pelleted diet and water was given *ad libitum.*

Parasites/antigen—*P. falciparum* strain was collected from infected human RBC (iRBC) by venipuncture on EDTA anticoagulant from Government General Hospital, Guntur, Andhra Pradesh. Then cells were washed thrice with Phosphate Buffered Saline (PBS) at 1000 rpm for 10 min. The supernatant serum was removed. The sedimented iRBC collected at the bottom of the test tube were removed with rubber bulb pipette. After obtaining iRBC, they were immediately inoculated into the experimental mice (300 µL/mouse ip). *P. falciparum.* antigen/iRBC which were not used immediately were stored at -20 ^{o}C until use.

Counting of parasitemia—Thin smears of peripheral blood samples were taken from the tails of the mice and stained with Leishmann's stain. Usually the ring forms of the parasite develop in the blood by third day after iRBC were injected. These blood smears were used for malaria parasite counting. Parasitemia in mice was expressed as the overall percentage of infected RBC in the peripheral blood. Thus, the course of infection was studied in all the experimental groups by counting the parasites everyday for 30 days serially. Slides were examined at least twice to record the number of *P. falciparum* parasites.

Antimalarial treatment—To validate the present model, the main antimalarial drugs used to treat *P. falciparum* infections, i.e., Arteether (EMAL, Themis Medicare Ltd., Haridwar) and Artesunate (Falcigo tablet, Cadila Healthcare Ltd., Solan) which are blood schizonticides were tested. Drugs were administered when parasitemia had been stable for at least 2 days and when ring forms were the predominant stage. For each antimalarial drug, the parasite clearance time (PCT) and parasitemia were determined after 5 days of treatment.

Arteether is commercially available in the form of intramuscular injection; each 2 mL ampoule of EMAL contains 150 mg of Arteether. Arteether was administered ip as 2 mg/kg once a day for 5 days, with a double divided dose administered on the first day. Artesunate (Falcigo tablet) is commercially available in the form of tablet, each containing 50 mg of Artesunate. Artesunate was administered orally (using a gastric cannula for delivery) as a suspension in sterile water, 2 mg/kg once a day for 5 days, with a double divided dose administered on the first day.

Experimental design—C57BL/6J mice were infected with *P. falciparum* (washed human iRBC

lodging *P. f.*) and blood smears were prepared daily for 30 days (from day 1 of infection) for identification of merozoites, trophozoites (rings stages), schizonts and gametocytes and for parasite counts. Experimental mice were divided into following 5 batches of 6 mice each: *P. f.* infected (Batch-1), *P. f.* infected + Arteether treated (Batch-2), *P. f.* infected + Artesunate treated (Batch-3), Arteether treated + *P. f.* infected (Prophylaxis) (Batch-4) and Artesunate treated + *P. f.* infected (Prophylaxis) (Batch-5). Another batch of mice was kept as Control (Batch-6). The course of infection was studied in all the experimental groups by counting the parasites and the results were expressed in terms of the number of infected cells/100 RBC. Parasite clearance time was also recorded in each experimental group. Thus the effect of each drug was revealed in the present study.

Results and Discussion

Course of infection of P. falciparum in C57BL/6J mice—The effect of artemisinin derivatives were widely documented in *P. falciparum* infected humans, providing a positive control with which to determine the value of the *P. f.* iRBC-C57BL/6J mice model for antimalarial chemotherapy[10-14].

In agreement with other findings[7,8], the present protocol led us to obtain consistent and sustained parasite growth in *P. f.* iRBC-C57BL/6J mice. Ring forms were often seen attached along the margin of the red cell (Fig. 1b). Double invasion and multiple invasions were commonly observed and parasite lies at various parts of the erythrocyte. The subsequent stages of a sexual cycle i.e., late trophozoite, early and mature schizonts were not ordinarily seen in peripheral blood smear, except in very severe malarial infection. Late trophozoites appeared on the surface of the infected red cells associated with knob like projections on the erythrocyte membrane. Along with, many merozoites were observed in the plasma.

During the course of infection, on first 2 days after inoculation of *P. falciparum* antigen, the parasites were absent. The infection first appeared on the 3rd day, gradually the parasitemia increased up to 9th day with 30%, on 10th day with 35%, on 11th day with 40%, on 12th with 46%, on 13th day with 48%, on 14th day with 49.2% and the high rate of parasitemia was seen between 9-14 days. The merozoites, ring forms and trophozoites were appeared. On 16th day parasitemia was 44%, on 17th day with 38%, on 18th day with 32%, later the infection gradually decreased and disappeared completely by 30th day (Fig. 2a).

Similar studies were made on 'course of infection' to *Plasmodium pinottii* in experimental chicks[15] where the peak level of infection appeared on day 10. In another report[16], 40% of parasitemia at peak level with *P. falciparum* appear to be similar to the present study.

In vivo antimalarial effect upon P. falciparum in P. f. iRBC - C57BL/6J mice—In the present study, the peak parasitemia was found to be 18% in Batch-2 (Arteether treated) and 16% in Batch-3 (Artesunate treated) mice on day 9 of infection. Then the parasitemia was gradually decreased to 16.8 and 14 on day 10, 12.2 and 8 on day 11, 9 and 4% on day 12, 4 and 2% on day 13 in both Batch-2 and Batch-3 respectively. It is of interest to note that in Batch-2 there was 2% of parasitemia on day 14 and no parasites found from day 15 to 30; in Batch-3 no parasites were found from day 14 to 30. The PCT was 7 days in Arteether group where as in Artesunate group parasites were killed one day (6 days) advance compared to arteether (Fig. 2a and b). Comparatively

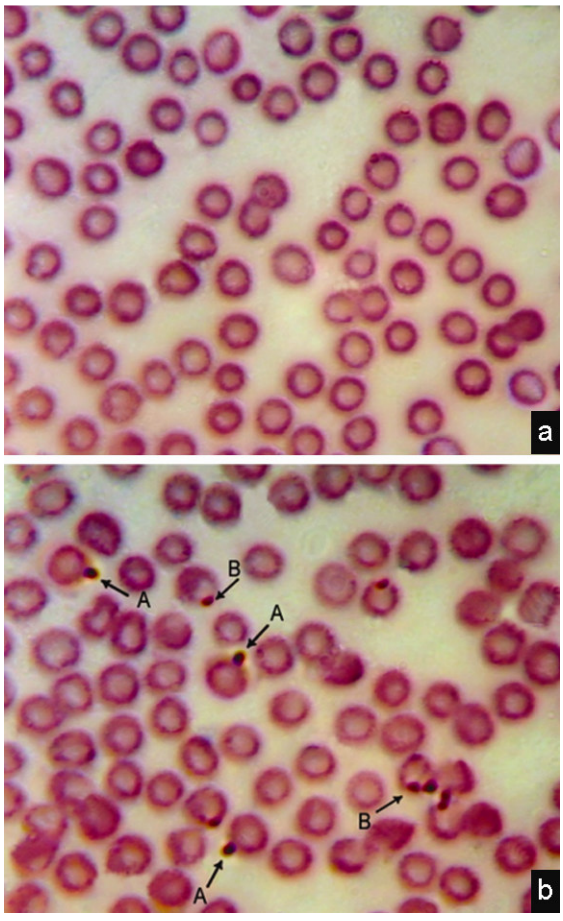

Fig. 1—Normal mouse cells without any infection (a) x100. Development of *P. falciparum* showing ring forms (b) x100.

the retained parasitic burden was less in Batch-3 mice. These drugs showed potent growth inhibitory activity of parasite against the multidrug-resistant *P. falciparum* strain. And no parasite recrudescence was observed in Arteether or Artesunate treated groups.

Batch-4 mice (Arteether treated + *P. f.* antigen infected) showed 2% of parasitemia on day 8, 1% on day 10 and parasites were completely disappeared on day 11 (Fig. 3a). Batch-5 mice (Artesunate treated + *P. f.* antigen inoculated) showed 1% parasitemia on day 8, 2% on day 9 and 1% on day 10 and total destruction of parasites on day 12 (Fig. 3b). Thus there is a rapid clearance of parasites with Arteether and Artesunate prophylaxis. The parasitemia observed was negligible when compared with the *P. f.* infected mice. The parasite clearance time (PCT) was 72 h (3 days) with 100% parasite clearance.

The present results show that the *P. f.* infected human RBC-C57BL/6J mice model, in which iRBC are inoculated into the mice, can be employed as a tool to evaluate *in vivo* responses to antimalarial drugs as demonstrated by blood schizonticidal effects of artemisinin derivatives[17]. After inoculation of mice with iRBC, double and multiple invasions were commonly observed in the peripheral blood smears. The development of *P. falciparum* erythrocytic stages in C57BL/6J mice model is an important aspect and paved the way to study *P. falciparum* under *in vivo* conditions. The morphological account of *P. falciparum* does not give any additional characters but the experimental study revealed some aspects for future pursue.

Delay in parasite clearance by Arteether in the present study confirms with that of the other studies[18,19] that delay in parasite clearance by all antimalarial drugs is associated with increasing parasitemia and hyperparasitemia and is thought to be contributory to drug treatment failure in *P. falciparum* to antimalarials including artemisinin derivatives and artimisinin combinations[20]. However the present results showed that both Arteether and Artesunate are more effective drugs in reducing parasitemia in experimental mice.

In vivo, artemisinin produces fast parasite and fever clearance, is well tolerated, and has an important role in the treatment of severe and cerebral malaria[10]. Hence, artemisinin derivatives like Arteether and Aresunate seem to act on dividing forms, and this is a

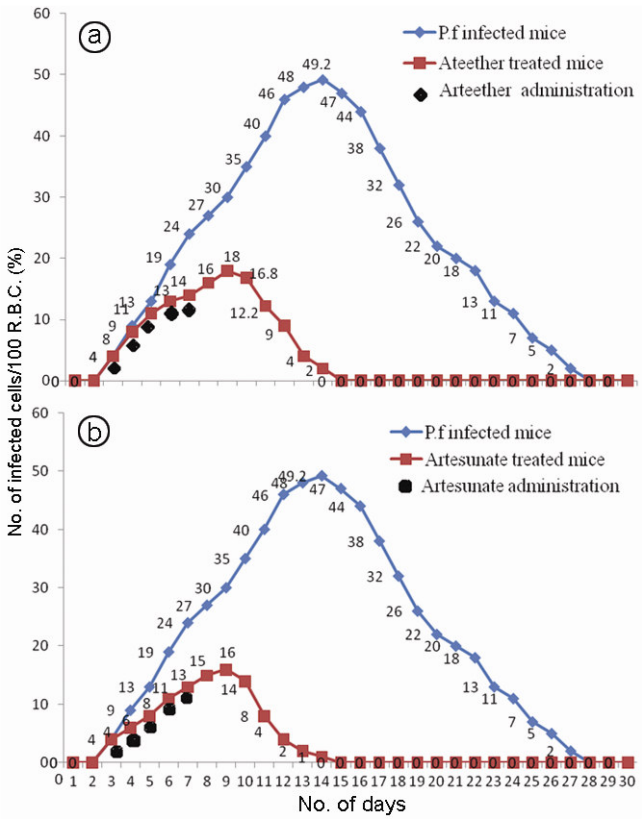

Fig. 2—Effect of (a) arteether and (b) artesunate on *P. falciparum*

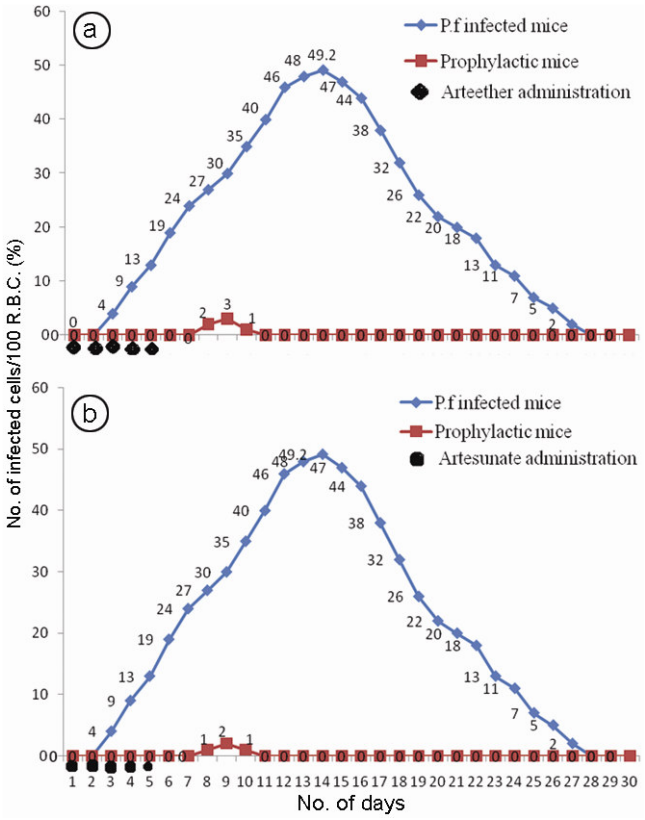

Fig. 3—Prophylactic treatment of (a) arteether (b) artesunate on *P. falciparum*

likely explanation for the absence of schizonts following Arteether or Artesunate treatment. This finding may be related to the fact that the drug can affect both protein synthesis and DNA replication[21], leading to schizont destruction. It was found that Arteether and Artesunate was highly effective against all stages of the parasites and observed that 24 h after the treatment of infected children with artemether, all of their parasites were abnormal[22,23]. Thus, the present data on the speed of clearance of targeted stage tend to validate the use of this model for chemotherapy studies of *P. falciparum* malaria.

The effect of antimalarial drug with regard to the reduction of parasitemia and PCT in the *P. f.* infected human RBC-C57BL/6J mouse model suggests that this model is valuable for studying the biology of human *Plasmodium* infections and immune responses to this parasite supported by Badell *et al.*[24]. In the present investigation the total clearance of parasitemia in the experimental mice by arteether and artesunate within seven and six days respectively, showed that the drugs are still effective against malarial parasites.

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