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# Effects of chloroquine treatment on circulating erythropoietin and inflammatory cytokines in acute *Plasmodium falciparum* malaria

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**Abstract** Anemia is a common and serious complication of malaria due to *Plasmodium falciparum* infection, a major health problem in tropical areas. Herein, the relation was investigated between the levels of circulating erythropoietin (EPO) and immunomodulatory cytokines in response to chloroquine treatment. Thirty-seven healthy control subjects and 40 patients with acute *P. falciparum* infection were included in the study. All subjects were adult male Sudanese. Blood samples were collected before chloroquine administration (25 mg/kg body weight, orally on three consecutive days) and 3 and 30 days after start of the therapy. Measurements included routine hematological parameters and the concentrations of immunoreactive EPO, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin 1 $\alpha$  (IL-1), IL-6, and interferon  $\gamma$  (INF- $\gamma$ ). Chloroquine treatment led to a decrease in EPO levels in the control subjects but an increase in malaria patients at day 30. The latter was likely due to the anti-inflammatory action of the drug because INF- $\gamma$ , IL-1, and IL-6 concentrations declined on chloroquine treatment. Based on these findings, we propose that an impaired EPO production in association with a prolonged elevation of certain inflammatory cytokines can contribute to the anemia in some malaria patients which can be reversed by chloroquine therapy.

**Keywords** *Plasmodium falciparum* · Erythropoietin · Malaria · Inflammation · Cytokines · Anemia

## Introduction

Malaria is a serious disease with globally  $3 \times 10^8$ – $5 \times 10^8$  new infections and 100 million deaths every year. The disease is responsible for about 10% of hospital admissions and 20–30% of outpatient consultations in sub-Saharan countries. In Sudan, malaria continues to cause high mortality and morbidity, particularly among children and pregnant women. Chloroquine was used in Sudan for over 40 years as a first-line treatment of malaria due to infection with *Plasmodium falciparum*, which is the most prevalent among the *Plasmodium* strains. In view of *P. falciparum* chloroquine-resistant strains, a switch to artemisinin-based combination therapy has been initiated only recently [13].

Several mechanisms contribute to the development of anemia in malaria, including the lysis of both parasitized and non-parasitized erythrocytes, bone marrow suppression, and dyserythropoiesis [12, 14, 19]. Erythropoiesis is inhibited until clearance of the parasites [11]. It is likely that cytokines are involved in the suppression of erythropoiesis. Serum concentrations of tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), interferon  $\gamma$  (INF- $\gamma$ ), interleukin 1 $\alpha$  (IL-1), IL-6, and IL-10 are often increased in acute *P. falciparum* malaria and, in some patients, remain elevated for 1–4 weeks after treatment [9, 16, 21]. TNF- $\alpha$ , IL-1, and INF- $\gamma$  inhibit the proliferation of erythrocytic progenitors [15] and heme synthesis [20]. IL-1 and TNF- $\alpha$  suppress erythropoietin (EPO) gene expression in vitro and in experimental animals [10]. In addition, studies from our own laboratory have shown that chloroquine at concentrations  $\geq 10$   $\mu\text{g/ml}$

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inhibits EPO production in the human hepatoma cell line HepG2 [6]. Whether chloroquine treatment affects EPO production in vivo is not known.

In the present study, the concentrations of blood hemoglobin (Hb) and serum EPO were measured in 40 Sudanese patients suffering from malaria due to *P. falciparum* infection and in 37 healthy control subjects before and 3 and 30 days after chloroquine intake. In addition, the effects of chloroquine treatment on the concentration of circulating IFN- $\gamma$ , TNF- $\alpha$ , IL-1, and IL-6 were assessed in the malaria patients.

## Materials and methods

### Area and population

The study was performed at Kenana Sugar Company Hospital in the Kenana area, which is located in the Savanna region between the White Nile and the Blue Nile, 350 km south from Khartoum, Sudan. The area is predominately hyperendemic for *P. falciparum*.

Sixty-one consecutive acute adult malaria patients of different socioeconomic status and age (16–44 years) were included in the study. Inclusion criteria were: (1) infection with *P. falciparum* only (no evidence of concomitant *Plasmodium vivax* on onset or during follow-up), (2) no history of malaria or application of antimalarial drugs for at least 3 months, (3) clearance of the asexual form of *P. falciparum* from the peripheral blood on chloroquine treatment within 3 days, and (4) no blood transfusion during the study period. Patients' complaints were consistent with symptoms of uncomplicated malaria, such as fever, nausea, headache, dizziness, and fatigability. Forty patients completed the full period of the study, while the others had to be excluded due to chloroquine intake failure or other reasons. Thirty-seven uninfected adult male volunteers participated as a group of healthy controls. None of them had a history of malaria, treatment with antimalarial drugs, or blood donation for at least 3 months. All participants were male Sudanese. They gave informed consent prior to enrollment in the study. The investigation was approved by the Research Committee of the Faculty of Medicine of the University of Khartoum.

Patients with malaria due to *P. falciparum* infection and the healthy control subjects received the standard dose of chloroquine earlier recommended by the WHO (25 mg/kg body weight, taken orally on three consecutive days).

### Samples

Blood samples were collected from patients and control subjects by venous puncture on day 0 (admission of

patients) and days 3 and 30 after commencement of chloroquine intake. Thick and thin blood smears were prepared from a finger prick for microscopic malaria diagnosis. Blood samples were prepared in two separate tubes. One contained EDTA, which was used to determine hematological parameters and to obtain plasma for the assay of cytokines. The other contained no anticoagulant and was maintained at 4°C overnight to obtain serum for the assay of EPO. Plasma for the assay of cytokines was not obtained from healthy subjects treated with chloroquine and not available from some of the malaria patients.

### Laboratory measurements

Routine examinations included Hb measurements, hematocrit (Hct), white blood cells counts, reticulocyte counts, creatinine and urea concentrations, and liver function parameters.

Serum EPO was measured by radioimmunoassay in duplicate using [<sup>125</sup>I]-labeled rhu-EPO (111–148 TBq/mmol; Amersham Buchler, Braunschweig, Germany) and antiserum from a rabbit previously immunized with rhu-EPO (Epoetin alfa; Ortho Biotech, Neuss, Germany). The EPO standard was calibrated by in vivo bioassay against the international reference preparation B. Mixtures of 100  $\mu$ l anti-EPO serum (1:3,000) and 100  $\mu$ l test sample or EPO standard (range 0–20 U/l) were incubated at 4°C for 48 h. Then, 100  $\mu$ l [<sup>125</sup>I]-labeled EPO was added for a further 24-h incubation at 4°C. Antibody-bound [<sup>125</sup>I]-EPO was separated from free [<sup>125</sup>I]-EPO using donkey anti-rabbit IgM (Amerlex M, Amersham Buchler). The lower detection limit of the assay was 2 U/l.

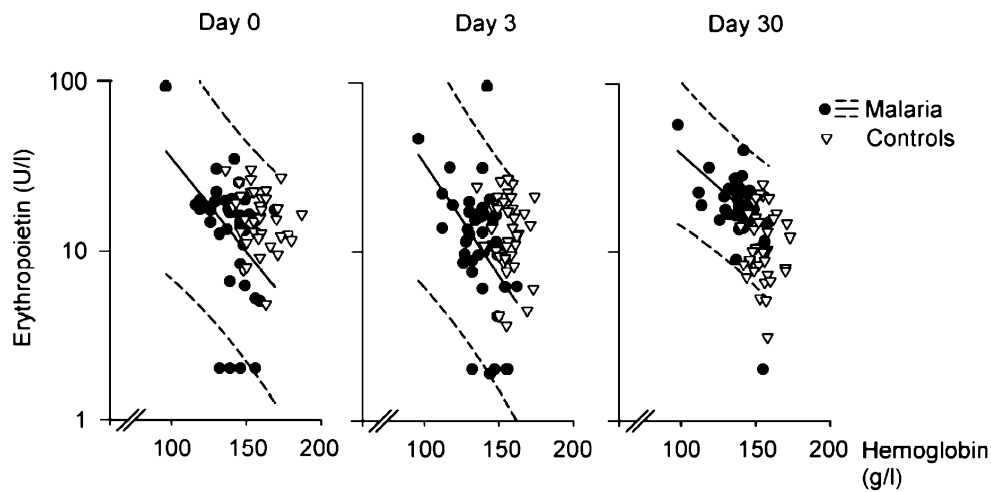
For assay of TNF- $\alpha$ , IL-1 $\alpha$ , IL-6, and INF- $\gamma$  in plasma, commercial enzyme-linked immunoassays were used exactly as described by the manufacturer (Diaclone Research, Besancon, Cedex, France).

### Statistics

Results were evaluated with the GraphPad InStat Software (San Diego, USA). Data are given as the median and/or the mean  $\pm$  SEM as indicated. Non-parametric tests (Dunn's multiple comparisons test for related samples, respectively, unrelated samples, Spearman rank correlation) were applied where appropriate. Significance was assumed at  $P < 0.05$ .

## Results

All control subjects presented with normal Hb values. Their median Hb concentrations were 159.0 g/l (mean  $\pm$  SEM, 158.9  $\pm$  1.9 g/l) on day 0, 156.0 g/l (156.0  $\pm$  1.4 g/l) on day 3, and 155.0 g/l (154.4  $\pm$  1.3 g/l) on day 30 after chloroquine



**Fig. 1** Semilogarithmic presentation of the relationship between serum immunoreactive EPO and blood hemoglobin levels in healthy male Sudanese subjects (*inverted triangle*,  $n=37$ ) and in malaria patients (*circle*,  $n=40$ ) before (day 0) and after chloroquine treatment (days 3 and 30). EPO concentrations below the detection limit were

set 2 U/l. Regression lines and 95% confidence intervals are shown for malaria patients only. Slopes of the regression lines were  $-0.011$  on day 0 ( $r=-0.43$ ,  $P=0.006$ ),  $-0.013$  on day 3 ( $r=-0.48$ ,  $P=0.002$ ), and  $-0.008$  on day 30 ( $r=-0.45$ ,  $P=0.003$ )

intake. Hb values at day 30 were significantly lower than at day 0 ( $P<0.001$ , Dunn's test). All of the 40 patients suffered from uncomplicated *P. falciparum* malaria and responded to the treatment with chloroquine. Parasite counts in blood were  $24,540\pm 5,270$  per microliter on day 0 and not detectable on day 3. The period of fever before diagnosis was  $2.3\pm 0.2$  days (mean $\pm$ SEM). Using the WHO criterion [22], the prevalence of anemia (Hb $<$ 130 g/l) was 20% (eight men) in the malaria patients before chloroquine treatment was started. Median Hb values were 140.0 g/l (mean $\pm$ SEM,  $139.3\pm 2.2$  g/l) on day 0, 139.0 g/l ( $136.8\pm 2.1$  g/l) on day 3, and 140.0 g/l ( $138.5\pm 1.9$  g/l) on day 30. Hb values at day 3 were significantly lower than at day 0 ( $P<0.05$ , Dunn's test). Leukocytosis was not apparent in the malaria patients even on day 0 before chloroquine treatment was started ( $4,125\pm 71$  leukocytes per microliter of blood, mean $\pm$ SEM).

The concentration of immunoreactive EPO in the sera of the control subjects ranged from 5 to 30 U/l before chloroquine treatment (day 0). Median EPO concentrations were 15.7 U/l (mean $\pm$ SEM,  $16.6\pm 1.0$  U/l) on day 0, 13.7 U/l ( $14.4\pm 1.0$  U/l) on day 3, and 10.3 U/l ( $12.2\pm 0.9$  U/l) on day 30. EPO concentrations at day 30 were significantly lower than at day 0 ( $P<0.001$ , Dunn's test). EPO concentrations in the malaria patients before treatment (day 0) ranged from  $<2$  to 93 U/l. Median EPO concentrations were 16.5 U/l (mean $\pm$ SEM,  $16.7\pm 2.3$  U/l) on day 0, 12.0 U/l ( $15.0\pm 2.5$  U/l) on day 3, and 19.6 U/l ( $20.5\pm 1.4$  U/l) on day 30. Thus, EPO levels were significantly reduced on day 3 but increased on day 30 after commencing chloroquine treatment ( $P<0.05$ , Dunn's test).

Figure 1 is a semilogarithmic presentation of the serum EPO concentration in dependence of the blood Hb concentration in the control subjects and the malaria

**Table 1** Concentration of circulating cytokines in untreated control subjects and in malaria patients before (day 0) and after chloroquine intake (days 3 and 30)

Cytokine (pg/ml)	Healthy control subjects ( $n=11-15$ )	Malaria patients ( $n=23-24$ )		
	Day 0	Day 0	Day 3	Day 30
TNF- $\alpha$	0 (4 $\pm$ 2)	41 (136 $\pm$ 37) <sup>a</sup>	61 (223 $\pm$ 64) <sup>a</sup>	38 (104 $\pm$ 45) <sup>a</sup>
IFN- $\gamma$	7 (6 $\pm$ 0.5)	8 (20 $\pm$ 6)	7 (8 $\pm$ 0.6) <sup>b</sup>	7 (6 $\pm$ 0.5) <sup>b</sup>
IL-6	18 (21 $\pm$ 4)	19 (29 $\pm$ 7)	13 (19 $\pm$ 7) <sup>b</sup>	14 (16 $\pm$ 4) <sup>b</sup>
IL-1 $\beta$	0 (1.3 $\pm$ 0.9)	2.6 (5.7 $\pm$ 2.7)	0 (1.7 $\pm$ 1.3) <sup>b</sup>	0 (1.4 $\pm$ 0.6) <sup>b</sup>

Values are medians with means $\pm$ SEM in parenthesis

<sup>a</sup>  $P<0.05$  compared to controls (Dunn's test for unrelated samples)

<sup>b</sup>  $P<0.05$  compared to malaria patients before chloroquine treatment (day 0, Dunn's test for related samples)

patients on days 0, 3, and 30. A significant correlation was assessed only in the diseased malaria patients, i.e., before and on day 3 after commencing chloroquine therapy (Spearman  $r=0.45$ ,  $P<0.005$ ).

Compared to the very low concentration of plasma TNF- $\alpha$  in the control subjects, TNF- $\alpha$  was increased in the malaria patients even after the administration of chloroquine (Table 1). In contrast, the concentrations of IFN- $\gamma$ , IL-6, and IL- $\beta$  decreased in the course of chloroquine treatment.

## Discussion

Malaria-induced anemia due to *P. falciparum* infection is of multifactorial origin. It involves red blood cell destruction and phagocytosis, hypersplenism, autoimmune mechanisms, inhibition of red blood cell production, and ineffective erythropoiesis [12, 14]. In asymptomatic malaria, hemolysis may be a major cause of anemia [18], while in acute malaria, suppression of erythropoiesis in association with the inflammatory response appears to play a major role. It was the aim of the present study to measure serum EPO concentrations in adult men suffering from acute *P. falciparum* infection and to explore the effects of chloroquine treatment on the concentration of circulating EPO and inflammatory cytokines.

The concentration of circulating EPO ranged from 5 to 30 U/l in healthy Sudanese men, which confirms data by El Hassan et al. [6]. The slightly reduced EPO levels in the control group on day 30 after chloroquine treatment was likely due to a direct inhibition of EPO synthesis. Earlier studies from our laboratory had shown that chloroquine at concentrations  $>3$   $\mu\text{g/ml}$  produces a dose-dependent decrease in EPO production in human hepatoma cells of the line HepG2, while no such inhibition was seen with pyrimethamine in combination with sulfadoxine [6]. Chloroquine also lowers EPO mRNA levels in HepG2 cells (unpublished observation). It can be assumed that chloroquine was still present at day 30 after the start of the 3-day therapy. Clearance of the drug is dose-dependent; its terminal half-life has been reported to increase from 3 h after intake of 250 mg to 312 h after 1,000 mg [8].

Burgmann et al. [3] and El Hassan et al. [6] have reported that EPO levels are relatively low in *P. falciparum*-infected adult malaria patients when compared to the one in non-infected control subjects. We observed an increase in the EPO level along with decreased IFN- $\gamma$ , IL- $\beta$ , and IL-6 concentrations at day 30 following chloroquine treatment. When Chang and Stevenson [4] measured the EPO content in renal extracts of mice infected with *P. chabaudi*, univariate analysis identified the hct value, but none of the single cytokines studied (TNF- $\alpha$ , IL-4, IL-10, IL-

12p70, GM-CSF) as a major variable determining the renal EPO level. Provided there is a normal EPO generating apparatus in the kidney, serum EPO levels increase exponentially as Hb concentrations decrease. This relationship was shown to be maintained in the present study, confirming observations in children with malaria due to *P. falciparum* infection [2, 11].

Investigators have studied serum cytokine levels in a variety of clinical settings in patients with malaria [9]. Such cross-sectional studies have not been designed to recognize preclinical or transient peaks in cytokines, several of which have extremely short serum half-life [1]. Our longitudinal study demonstrated the initially greatly increased concentration of TNF- $\alpha$  in the blood of malaria-infected patients. TNF- $\alpha$  is thought to be produced rapidly in response to parasitized erythrocytes or lipopolysaccharide stimulation and induces the elaboration of a cascade of additional mediators in the cytokine network [7].

The present study included only patients who responded to chloroquine therapy. Chloroquine resistance has increased in the Sudan since the 1970s. Unresponsiveness was found to be 75.9% in Gadaref in eastern Sudan and 32.1% in Haj Yousif of the Khartoum state [17]. The differences in the clinical response following treatment with chloroquine are likely due to elements of the host immunity and the prevalence of *P. falciparum* DNA polymorphisms [17]. Sudanese health authorities have updated malaria treatment in 2004 from chloroquine to artemisinin-based combination therapy (ACT) using the co-blister of artesunate + sulfadoxine/pyrimethamine and artemether + lumefantrine as first- and second-line, respectively [5]. Effects of the novel therapy regimen on erythropoiesis and red blood cell status still need to be clarified. In addition, since chloroquine is widely available and cheap, many care givers still prefer it. Sudan moved from chloroquine to ACTs without an interim period, in contrast to other countries in Africa. A recent survey carried out in ten states has shown limited availability of ACTs, with treatment of malaria still relying on chloroquine [13].

In conclusion, chloroquine therapy will result in increased EPO levels in association with its anti-inflammatory action, although the drug can per se inhibit EPO synthesis.

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