

DEFECTIVE ERYTHROPOIETIN PRODUCTION AND RETICULOCYTE RESPONSE IN ACUTE *PLASMODIUM FALCIPARUM* MALARIA-ASSOCIATED ANEMIA

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Abstract. To elucidate the relationship between falciparum malaria-associated anemia and serum erythropoietin (Epo) levels and reticulocyte response during acute malaria infection, 87 adults aged 18-65 years presenting with acute, uncomplicated malaria were examined on enrollment and for 28 days of follow-up. The 87 patients were divided into 2 groups: those with anemia ($n = 45$) and those without ($n = 42$). Serum samples were taken on admission (Day 0), then on Days 7, 21, and 28, to measure the reticulocyte count, absolute reticulocyte count, reticulocyte hemoglobin content, and erythropoietin level (Epo). The absolute reticulocyte counts for the anemic patients were significantly higher than for those without anemia on Days 0, 7, 21, and 28. The serum Epo levels for the anemic patients were significantly higher than the non-anemic group only on Day 0 (44.39 ± 4.06 vs 25.91 ± 4.86 mIU/ml, $p < 0.001$). Inadequate Epo production was found in 31.03% (27/87) of patients on Day 0, 37.93% (33/87) on Day 7, 43.67% (38/87) on Day 21, and 39.08% (34/87) on Day 28. These results indicate defective Epo production and reticulocyte response in adult patients suffering from acute *P. falciparum* malaria, which differs from pediatric patients. Our findings may provide the basis for further study into the choice of therapeutic strategies to treat acute *P. falciparum* malaria-associated anemia with recombinant human Epo to correct refractory anemia due to malaria.

INTRODUCTION

Anemia is a common, life-threatening complication of *Plasmodium falciparum* malaria. The pathophysiologic background of anemia in malaria is complex and multifactorial (Woodruff *et al*, 1979; Phillips and Pasvol, 1992; Menendez *et al*, 2000). The major contributing factors include hemolysis of both

parasitized and non-parasitized red blood cells. A depressed erythropoietic response also contributes to the development of anemia (Dormer *et al*, 1983; Yap and Stevenson, 1994). Why some malaria patients develop severe anemia while others retain near-normal hemoglobin levels may be explained by the degree of red-cell destruction during the period before normal bone-marrow function returns. Anemia may either develop rapidly with severe hemolysis, or present as a relatively slow rate of red-cell destruction in the presence of persistent bone-marrow suppression. There are numerous studies regarding the duration and mechanism of bone-marrow suppression during malaria infection; some studies have

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demonstrated evidence of hypoproliferative erythropoiesis and dyserythropoiesis for weeks post-infection, while others found that bone-marrow suppression reverses rapidly after treatment (Burchard *et al*, 1995; Burgmann *et al*, 1996; el Hassan *et al*, 1997; Kurtzhals *et al*, 1997; Chang *et al*, 2004a).

During acute malaria infection, reticulocyte release is delayed, indicating transient suppression of the normal erythropoietin (Epo) response. The effect is probably mediated by an autologous serum factor that suppresses the growth of an early-precursor red cell (Roberts *et al*, 2005). Serum Epo has been found to be elevated in some African children with severe malaria-related anemia (Burchard *et al*, 1995; Verhoef *et al*, 2002), but in Thai, Sudanese, and Kenyan adults with malaria, serum Epo, although elevated, was not as high as might have been expected based on the degree of anemia (Burgmann *et al*, 1996; el Hassan *et al*, 1997; Vedovato *et al*, 1999). It is possible that inflammatory mediators, such as tumor necrosis factor (TNF) or IL-10, suppress Epo synthesis in adults with anemia and respond with inappropriately low numbers of reticulocytes.

Bone-marrow studies have indicated depressed erythropoiesis in cases of malaria. These dyserythropoietic changes included multinucleated erythroblasts, nucleus rupture, and disintegration of chromatin (karyorrhexis), as well as incomplete mitosis (Abdalla *et al*, 1980). Kinetic studies have demonstrated a high turnover in polychromatic erythroblasts (Dormer *et al*, 1983). The cause of this dyserythropoiesis has not yet been elucidated. The release of interferon gamma (IFN- γ) and TNF in the bone marrow can inhibit hematopoiesis (Yap and Stevenson, 1994). Sinusoidal obstruction by parasitized red cells may lead to bone marrow hypoxia and dysfunction (Abdalla, 1990). Dyserythropoiesis also occurs in the bone marrow of patients with low parasitemia. Kidney involvement in ma-

laria may lead to decreased erythropoietic production. Anemia is more severe in malaria patients with renal impairment; acute renal failure, frequently found in nonimmune adults with falciparum malaria, can result in reduced Epo production.

To clarify the relationships between reticulocyte response, serum-Epo levels and anemia during acute *P. falciparum* infection, a longitudinal study was conducted on adults presenting with malaria-related anemia. The anemia during the first 4 weeks after effective treatment was monitored. Erythropoietic response was evaluated by serum concentration of Epo and absolute reticulocyte count.

MATERIALS AND METHODS

Study population

The study population consisted of blood samples obtained from 87 adult males and non pregnant females aged 18-65 years, presenting with acute *P. falciparum* infection at the Hospital for Tropical Diseases, Mahidol University, Thailand, during March 2005-September 2006. The patients were admitted to the Hospital for 4 weeks following the initiation of antimalarial therapy. The exclusion criteria for sample recruitment were: pregnant and nursing women, individuals with signs and symptoms of severe malaria, mixed malarial infection on admission, current drug therapy for malaria, blood transfusion in the previous 30 days, laboratory evidence or history of significant cardiovascular, hepatic or renal abnormalities, and reported alcohol or drug abuse. Written informed consent was obtained from all study participants and the protocol was approved by the relevant institutional review boards (IRB).

Baseline studies

Age, sex, body weight, duration of fever pre-enrollment, liver and spleen size were recorded. A complete blood count (CBC), absolute reticulocyte count and reticulocyte

hemoglobin content (CHr) count were conducted using an automated cell counter (Advia 120 Bayer, Germany). A fingerprick blood sample was taken to prepare thick and thin blood films, and stained with Field's stain. Peripheral blood concentrations of asexual forms of *P. falciparum* were estimated either by counting the number of asexual forms per 200 WBC on thick smears and multiplying by the WBC count, or by counting the number of asexual forms per 1,000 RBC on thin smears and multiplying by the RBC count. Serum concentrations of total bilirubin, direct bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN), and creatinine were measured by Integra 400 (Roche Diagnostic, Switzerland). Serum Epo levels were measured by automated two-step enzyme linked immunosorbent assay (ELISA) (IBL, Hamburg, Germany). The normal range for the assay is 15-40 mIU/ml. The patients were divided into 2 groups, based on Hb concentration; for males, a Hb < 14 g/dl was designated as anemic, and for females a Hb < 12 g/dl.

Follow-up studies

Body temperature was recorded every 6 hours. Parasite concentrations were estimated every 12 hours, until the patient became aparasitemic and, thereafter every 24 hours. A CBC, biochemical analysis, serum Epo, absolute reticulocyte count and CHr were obtained on Days 7, 21, and 28 post-initiation of treatment.

Statistical analysis

All data were logarithmically transformed to achieve normal distribution. Differences between groups were analyzed using the two-tailed Student's *t*-test or Mann-Whitney two-sample rank-sum test. Associations between parameters were analyzed by multiple regression analysis. P-values < 0.05 were considered statistically significant. All calculations were performed using SPSS 11.0 (Chicago, USA).

RESULTS

Eighty-seven patients were enrolled in the study, consisting of 45 anemic and 42 non-anemic patients whose clinical characteristics are shown in Table 1. Severe anemia (Hb < 5 g/dl) was found in 1 of 45 patients (2.22%), moderate anemia (Hb 5-8 g/dl) in 2 patients (4.44%), and mild anemia (Hb > 8 g/dl) in 42 patients (93.34%). A reticulocyte count, absolute reticulocyte count, CHr, and serum Epo were obtained on Days 0, 7, 21, and 28 (Table 2). Upon enrollment there were significant differences in reticulocyte counts, absolute reticulocyte counts, CHr, and serum Epo between the 2 groups. Reticulocyte and absolute reticulocyte counts increased significantly from baseline to Day 21 in both anemic and non-anemic patients, and decreased slightly on Day 28 post-admission. The CHr in the anemic patients increased significantly from baseline to Day 21, and decreased slightly on Day 28, but in non-anemic patients, CHr decreased from baseline to Day 21 and increased slightly on Day 28. This means that in non-anemic malaria patients, more young reticulocytes were released from the bone marrow into the blood circulation than in anemic patients. The serum Epo concentrations were significantly higher in the anemic group than the non-anemic group only on Day 0. On Days 7 and 21 the serum-Epo concentrations of the 2 groups were not significantly different. In the anemic patients, serum-Epo levels were slightly higher than normal levels on Days 0 and 7. This shows that in acute *P. falciparum* malaria the associated anemia is not sufficient to stimulate Epo.

Serum Epo levels were expressed in relation to hematocrit according to the methods of Cazzola and Beguin (1992) by determining the exponential regression of serum Epo versus hematocrit in reference subjects (Beguin *et al*, 1993) (Fig 1). The two least-squares regression equations for the serum

Table 1
Baseline characteristics of the study population.

	Anemic patients (n = 45)	Non-anemic patients (n = 42)
Age (years)	26.95 ± 8.60	25.26 ± 6.65
Sex (M:F)	38:7	37:5
Hb (g/dl)	10.85 ± 1.70	13.96 ± 2.32
Hct (%)	32.77 ± 5.27	41.90 ± 4.86
RBC (x10 ⁶ /μl)	4.22 ± 0.82	5.21 ± 0.56
Platelet (x10 ³ /μl)	100.93 ± 70.42	73.15 ± 52.24
RDW	14.76 ± 1.34	14.22 ± 1.62
Reticulocyte (%)	2.15 ± 1.59	1.13 ± 0.60
Absolute reticulocyte count (x 10 ⁹ /l)	82.57 ± 49.63	57.69 ± 24.72
CHr (pg)	27.20 ± 6.38	29.23 ± 11.26
Parasite count (x 10 ³ /μl)	78.53 ± 19.14	93.04 ± 33.56
Direct bilirubin (mg/dl)	1.16 ± 0.32	0.77 ± 0.15
Total bilirubin (mg/dl)	2.26 ± 0.42	2.04 ± 0.25
AST (IU/l)	41.04 ± 6.87	41.00 ± 5.19
ALT (IU/l)	36.15 ± 5.21	39.53 ± 5.52
BUN (mg/dl)	18.06 ± 1.41	16.77 ± 0.93
Creatinine (mg/dl)	0.89 ± 0.04	1.13 ± 0.24

Epo and Hct were computed, one for a Hct < 40% and the other for a Hct > 40%. This Hct cutoff was chosen because it allowed for the best correlation of serum Epo levels and because the literature indicates that in a Hct < 40%, there is little change in serum Epo level (Beguin *et al*, 1993). Comparing Hct levels with serum Epo concentrations we found an inadequate Epo response in 31.03% (27/87) of patients on admission day, 37.93% (33/87) on Day 7, 43.67% (38/87) on Day 21, and 39.08% (34/87) on Day 28 (Fig 2). The mean ± SD for Hct was 37.07 ± 6.82% on admission day, 34.89 ± 5.07% on Day 7, 37.23 ± 4.44% on Day 21, and 38.30 ± 4.27% on Day 28.

DISCUSSION

The mechanisms of malaria-associated anemia are complex and not completely understood. Potential contributing factors include the destruction of parasitized erythro-

cytes as a consequence of parasitic schizogony and the elimination of parasitized and nonparasitized erythrocytes in concert with immune and phagocytic mechanisms. Inhibited erythrocyte production and ineffective erythropoiesis have been investigated and are associated with anemia in *P. falciparum* infection (Phillips and Pasvol, 1992). The data reported here show clearly that the rate of erythrocyte destruction exceeds the rate of erythropoiesis. Although erythropoiesis among these patients was enhanced, as shown by the nearly double normal reticulocyte count throughout the course of infection, a definitive response via erythrocyte production was not possible since the circulating life-span of reticulocytes in malaria-associated anemia was not available (Table 2). The data from this study show that reticulocyte response may reflect an underestimation of the extent of erythropoiesis. It also indicates that erythropoiesis during falciparum malaria infection is

Table 2

Reticulocyte count, absolute reticulocyte count, reticulocyte Hb content, and serum Epo on Days 0, 7, 21 and 28 for anemic and non-anemic patients.

Laboratory parameter	Anemic (n = 45)	Non-anemic (n = 42)	p-value
Reticulocyte count (%)			
Day 0	1.96 ± 0.20	1.05 ± 0.32	<0.001
Day 7	3.03 ± 0.37	1.10 ± 0.10	<0.001
Day 21	3.25 ± 0.23	3.86 ± 1.69	NS
Day 28	2.67 ± 0.20	1.75 ± 0.11	<0.001
Absolute reticulocyte count (x10 ⁹ /l)			
Day 0	78.83 ± 6.52	55.12 ± 3.30	<0.001
Day 7	108.56 ± 10.63	52.30 ± 5.11	<0.0001
Day 21	140.97 ± 8.75	101.22 ± 9.30	<0.001
Day 28	120.46 ± 4.60	88.38 ± 5.46	<0.01
Reticulocyte Hb content (CHr) (pg)			
Day 0	26.80 ± 0.79	30.88 ± 2.50	<0.05
Day 7	28.43 ± 0.52	29.86 ± 0.60	<0.05
Day 21	29.30 ± 0.44	28.67 ± 1.66	NS
Day 28	28.70 ± 0.40	30.56 ± 0.70	<0.05
Serum Epo (mIU/ml)			
Day 0	44.39 ± 4.06	25.91 ± 4.86	<0.001
Day 7	41.40 ± 3.01	33.81 ± 4.39	NS
Day 21	36.01 ± 2.83	29.80 ± 4.71	NS
Day 28	28.33 ± 2.23	21.14 ± 3.21	<0.05

inadequate since the expected rise in peripheral reticulocytosis during the ensuing anemia does not occur until adequate antimalarial treatment has been administered. As shown in Table 2, the absolute reticulocyte count of anemic patients increased dramatically on Days 7 and 21 (from 78.83 to 108.56 on Day 7, and 140.97 x 10⁹/l on Day 21), however, the serum Epo levels of these patients did not increase in a similar fashion.

Hematopoiesis and the immune system have an interrelated network of growth factors, cytokines and interleukins regulating the proliferation and differentiation of cells in the network. Some of these factors have been shown to inhibit erythropoiesis *in vitro* and *in vivo* (Nussenblatt *et al*, 2001; Lyke *et al*, 2004). These include interleukin 1 (IL-1), interferon- γ

(IFN- γ), and tumor necrosis factor- α (TNF- α). Both IL-1 and TNF- α have previously been shown to lower Epo mRNA levels and to inhibit Epo formation in human hepatoma cell cultures, and to suppress Epo formation in isolated rat kidneys (Miller *et al*, 1989; Clark *et al*, 2006). TNF- α has also been shown to inhibit erythropoiesis *in vivo* and to increase erythrophagocytosis and dyserythropoiesis in the bone marrow of malaria patients.

In the laboratory diagnosis of anemia, serum-Epo concentrations should not be evaluated in absolute terms, but should rather be interpreted in relation to the degree of anemia. This will provide information on whether the Epo-generating cells in the kidneys are responding efficiently or inadequately to the degree of anemia as the hematocrit

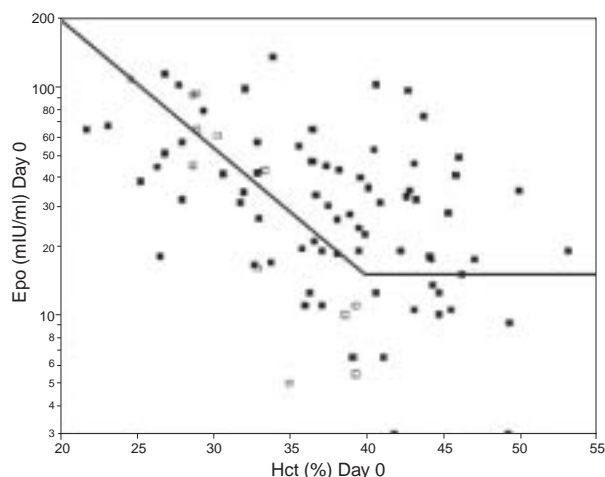


Fig 1—Relationship between Epo level and Hct in 87 patients with acute *P. falciparum* infection on admission day (Day 0). The regression line displays the relationship for reference subjects (■ = male, □ = female).

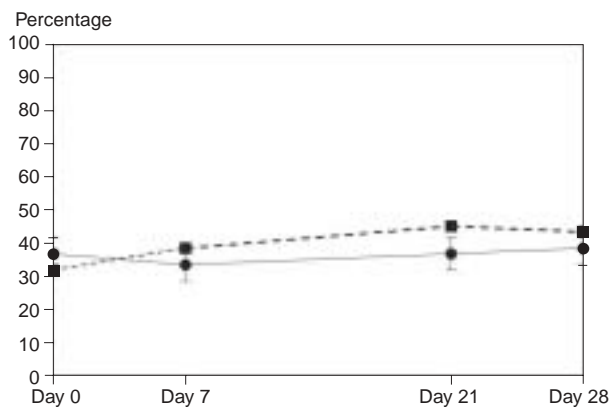


Fig 2—Hematocrit (% mean \pm SD) (●) and percentage of patients with defective serum Epo production (■) in patients suffering from acute *Plasmodium falciparum* infection ($n = 87$).

decreases. Thus, serum Epo concentrations must be expressed in relation to hematocrit levels. Epo production is typically suppressed in chronic renal failure (CRF) because of damage to the Epo-generating cells. In addition, inadequate Epo production for the degree of

anemia has been reported, such as in rheumatoid arthritis (RA) (Baer *et al*, 1987), acquired immunodeficiency syndrome (AIDS) (Spivak *et al*, 1989), and congestive heart failure (CHF) (Opasich *et al*, 2005). Some investigators have postulated that blunting of renal oxygen sensors is responsible for the anemia of RA (Baer *et al*, 1987). However, some cytokines play a major role in the development of anemia in these patients.

In the present study, we addressed defective serum Epo production in patients with acute *P. falciparum* infection. On admission day almost 31% of patients had inadequate Epo production and an inappropriate reticulocyte response, although serum Epo levels and absolute reticulocyte counts were elevated. On Day 7, around 38% had inadequate Epo production and an inappropriate reticulocyte response, and the rate continued to increase to 43% on Day 21, then decreased to 39% on Day 28 post-admission. Inadequate production of Epo and inappropriate reticulocyte response may contribute to prolonged anemia in these patients. Our results correlate with previous studies (Burgmann *et al*, 1996; el Hassan *et al*, 1997; Camacho *et al*, 1998; Vedovato *et al*, 1999; Casals-Pascual *et al*, 2006), but were not comparable with some pediatric studies (Burchard *et al*, 1995; Kurtzhals *et al*, 1997; Nussenblatt *et al*, 2001; Verhoef *et al*, 2002; Helleberg *et al*, 2005). The reason for the different results is attributable to the better ability of children's kidneys to produce the necessary Epo for regulating red blood cell production. Therefore, we suggest that investigators not compare Epo concentrations and reticulocyte responses in children and adults. The use of Epo for adult patients with anemia-associated falciparum malaria does not mean it can be used in pediatric patients. In 2004, Chang *et al* (2004b) reported the use of synthetic Epo in treating malaria-associated anemia and showed the important role of Epo-induced reticulocytosis

in modulating the course and outcome of blood-stage malaria in mice. The literature contains no similar studies in humans.

Besides the well-known hematopoietic role of Epo (stimulation of erythropoiesis), significant anti-inflammatory, anti-oxidant, and anti-apoptotic effects in various brain disorders have also been established. In 2006, Kaiser and colleagues demonstrated that Epo was one of the most promising drugs for treating cerebral malaria (CM) (Kaiser *et al*, 2006). They measured the effect of Epo on the survival of mice infected with *P. berghei* ANKA, and showed that inoculations of recombinant human Epo at the start of clinical manifestations could protect 90% of mice from a fatal outcome. Epo does not affect the course of parasitemia. The effect of Epo was not related either to inhibition of apoptosis in the brain nor regulation of the increase/decrease in nitric oxide production in the brain and blood, respectively. Recently, Casals-Pascual *et al* (2008) showed high levels of Epo were associated with reduced risk of neurological sequelae in children with CM. The age-dependent Epo response to anemia, and the age-dependent protective effects, may influence the clinical epidemiology of CM. Another study using a murine model showed that recombinant human Epo increased survival and reduced neuronal apoptosis in cerebral malaria (Wiese *et al*, 2008). These data support further study of Epo as adjuvant therapy in malaria-associated malaria and CM. These results may provide a basis for further research into the application of recombinant Epo in therapeutic strategies, such as Epo substitution in these groups of patients.

We conclude that acute, uncomplicated *P. falciparum* infection leads to defective Epo production and inappropriate reticulocyte response in adult patients. Further studies are needed to establish an effective treatment strategy for falciparum malaria-associated anemia using recombinant human Epo.

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