Severe malarial anemia associated with increased soluble Fas ligand (sFasL) concentrations in Gabonese children

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ABSTRACT. To investigate if severe malarial anemia is associated with specific cytokine overproduction, we evaluated serum levels of soluble Fas ligand (sFasL), tumor necrosis factor (TNF-α) and interleukin-10 (IL-10) from three groups of young children with *Plasmodium falciparum* infection (asymptomatic cases, uncomplicated malaria cases and severe malarial anemia cases), in a hyperendemic area of Gabon. In uncomplicated cases, only TNF levels were significantly ($p < 0.001$) increased in comparison to asymptomatic cases with *P. falciparum* infection. High levels of sFasL, TNF-α and IL-10 were associated with low hemoglobin concentrations, sFasL levels were significantly higher in children with severe malarial anemia ($p < 0.001$) as compared to both other groups. The parasite density was positively correlated with IL-10, TNF-α and sFasL levels. TNF-α and sFasL, but not IL-10 or parasitemia, were independent predictors of hemoglobin concentrations. These results suggest that, in malaria, a specific dysregulation of the cytokine balance may lead to complications such as severe anemia.

Keywords: *P. falciparum*, severe anemia, IL-10, TNF-α, children, Central Africa

INTRODUCTION

Malaria presents a wide spectrum of manifestations ranging from asymptomatic infections to life-threatening disease. Infections with the malaria parasite *Plasmodium falciparum* are responsible for the death of more than one million of children per year [1]. One of the most important complications of the disease contributing to mortality is severe malarial anemia. The pathogenesis of malarial anemia is complex and may vary from case to case depending on patient history, parasite density, degree of illness, and hemoglobin concentration. There is evidence that hemolysis occurs with both parasitized and nonparasitized erythrocytes, however, the contributions of precise factors involved in autoimmune hemolysis or other immune-mediated events in this phenomenon remain unclear. It has been suggested that suppressive, autologous serum factors, reduced erythropoietin synthesis, and alterations in cytokine production may contribute to the development of anemia [2].

The tumor necrosis factor-alpha (TNF-α), an inflammatory cytokine has been implicated in several cellular and biological changes associated with acute malaria, including stimulation of nitric oxide production, enhancement of the production of other cytokines, and inhibition of erythropoiesis [3-5]. TNF-α is down-regulated by interleukin-10 (IL-10), an anti-inflammatory cytokine, which appears to stimulate erythropoiesis [6]. TNF-α may have beneficial effects in malaria, while high TNF-α levels are associated with severe disease such as cerebral malaria or severe anemia [3]. The dysregulation of TNF-α and IL-10 could contribute to the pathogenesis of severe malarial anemia [7].

Fas ligand (FasL) is a type II membrane protein that belongs to the TNF-α and CD40 ligand family. Serum from patients with acute *P. falciparum* malaria contain elevated levels of sFasL [8]. sFasL induces apoptosis in susceptible cells after cross-linking to the sFasL receptor. It has been reported that lymphocyte apoptosis is higher in malaria patients compared to healthy individuals [9]. However, information on the role of soluble Fas ligand (sFasL) in malaria is limited. These observations underscore the need to better understand the role of immuno-regulator molecules such as sFasL and TNF-α that are of importance in both malaria immunity and/or pathology.

The impact of *P. falciparum* parasite density in the development of severe anemia is not entirely clear [2], however, there is some consensus on the importance of the IL-10/TNF-α ratio in severe malarial anemia, even though controversial data on the origin (low IL-10 and/or high TNF-α levels) of the cytokine balance exists [4, 5, 10, 11]. In Lambaréné (Gabon), severe anemia due to malaria is a major cause of hospitalisation at the Albert Schweitzer Hospital. We have undertaken a cross-sectional study in infants and young children with asymptomatic infections, uncomplicated malaria and severe malarial anemia in order; i) to investigate the association between the *P. falci-*

parum parasite density, hemoglobin concentrations, IL-10, sFasL and TNF-α levels, and ii) to determine which of these immunological factor has the strongest association with the hemoglobin concentration. Our objectives were to provide more information on parasitological and immunological parameters contributing to severe anemia, and, in particular to evaluate the role of sFasL as an important inflammatory mediator in the development of severe malarial anemia in children living in this area of Central Africa.

PATIENTS AND METHODS

Study area and population

The study was carried out in Lambaréné (Province of Moyen Ogooué, Gabon), where malaria transmission is intense and stable [12], with 23-61 infectious bites per person per year [13]. *P. falciparum* accounts for about 90% of all detected infections. *Anopheles gambiae* and *Anopheles moucheti* are the main vectors involved in the malaria transmission reported in this area [13]. The recruitment was done at the Albert Schweitzer Hospital. Enrolled children with severe anemia and aged less than five years old, were matched for age, sex and place of residence, with children with asymptomatic infection and uncomplicated malaria. Children of either sex, aged 6-57 months and residing in the vicinity of the Albert Schweitzer Hospital were recruited.

Group 1. Children with asymptomatic infections. All children had positive microscopy for *P. falciparum* infection, with less than 20,000 asexual blood stage parasites/µl of blood, a tympanic temperature < 37.5 °C on the day of recruitment and no history of fever 24 hours before and one week after the day of recruitment. Hemoglobin levels were above 10 g/dL and/or hematocrit > 30%. *Group 2*. Children with uncomplicated malaria and a tympanic temperature ≥ 37 °5, *P. falciparum* infection less than 250,000 asexual blood stage parasites/µl of blood and hemoglobin level > 5 g/dL and < 10 g/dL. Children in this group did not present any concomitant infections that might have induced fever or clinical symptoms, and did not have severe malaria as defined by WHO [14]. *Group 3*. Children with severe malarial anemia who were hospitalised in the pediatric ward for fever (temperature ≥ 37 °5), and severe anemia (Hb ≤ 5 g/dL or hematocrit < 15%), associated with a blood smear positive for *P. falciparum*.

Ethical considerations

Informed consent was obtained from each patient's parent or guardian and the study was approved by the ethics committee of the International Foundation of the Albert Schweitzer Hospital in Lambaréné, Gabon.

Blood collection

Venous blood was collected in heparin tubes at day 0 (the day of admission, immediately before antimalarial treatment). An aliquot of blood (500 µl) was used to determine hematological parameters. Plasma was separated after centrifugation of heparinized blood and stored at – 80 °C until cytokine assays were performed. The parasite density in blood was determined using Planche *et al.* (2001) methodology [15].

Cytokine assays

Blood concentrations of sFasL (OptiEIA, Pharmingen, Heidelberg, Germany), TNF-α (Caltag Laboratories, Hamburg, Germany) and IL-10 (Flexia, Biosource, Ratingen, Germany) were measured with commercially available enzyme linked immunosorbent assay (ELISA) kits. Detection limit was defined as 1 pg/mL for all three cytokines, and values below this level were assigned a value of 0. The optical density of each well was measured at 450 nm by a dual-wavelength plate reader (Mikrowin, Ortvath, Germany). The correlation between optical density ranging from 1 to 1 000 pg/mL was always highly significant ($r > 0.9$, $p < 0.01$).

Data analysis

For statistical analysis of data the Stata statistical software (release 8) was used. Spearman's correlation coefficients were calculated to assess associations between sFas levels, clinical status, parasitemia, IL-10/sFasL and IL-10/TNF-α ratios. Cytokine levels were compared using the Kruskal Wallis equality of population rank test. In addition, a stepwise linear regression model was developed to assess the impact of TNF-α, Il-10, sFasL and parasitemia on hemoglobin concentrations.

RESULTS

Characteristics of patients

Ninety two subjects were included in this study as followed: 32, 30, 30 in Group 1 (asymptomatic infections), Group 2 (uncomplicated malaria) and Group 3 (severe malarial anemia) respectively. Table 1 shows the parasitological and hematological characteristics of the patients on the day of recruitment. All the children were slightly malnourished based on the weight for age z score (weight

Table 1
Characteristics of children with *P. falciparum* infections

	Asymptomatic	Uncomplicated	Severe anemia	*P value*
Number of children	32	30	30	
Median age in months (range)	20 (6-57)	18 (6-78)	18 (6-54)	
Sex F/M	16/16	15/15	17/13	
Temperature (°C), (SD)	37.0 (0.2)	37.9 (0.8)	38.4 (0.8)	< 0.001
Median parasite density (range)	850 (120-10,800)	28,000 (2,100-185,000)	30,000 (300-960,000)	< 0.001
Hemoglobin (g/dL)	11.1 (1.0)	8.8 (1.7)	4.0 (0.7)	< 0.001

Note: SD Standard deviation.

Table 2
Cytokine levels in serum samples from children in the 3 groups

Cytokines (pg/mL)	Asymptomatic children	Uncomplicated malaria	Severe malarial anemia	*P value* [a]
IL-10	181 (130-220)	153 (49-850)	472 (105-960)	0.002
TNF-α	68 (39-110)	125 (68-250)	220 (230-1 000)	< 0.001
sFasL	402 (0-690)	332 (220-690)	1 240 (440-2 500)	< 0.001
IL-10/TNF-α	2.5 (0-4)	1.3 (0.5-7)	2.3 (0.3-5)	0.06
IL-10/sFasL	0.4 (0.2-3)	0.5 (0.1-3)	0.4 (0.07-1)	NS[b]

Note: All data are expressed as median (interquartile range):
[a] *P value*, comparison between the three groups.
[b] NS: non-significant.

for age) ←2 (data not shown). The hemoglobin concentrations, were significantly lower and temperature was significantly higher in the severe anemia group. The concentration of hemoglobin was negatively correlated with the parasite density (rho = – 0.49, $p < 0.001$).

IL-10 concentrations

As shown in Table 2, the highest levels of IL-10 were detected in the severe malarial anemia group ($p < 0.001$).

TNF-α concentrations

TNF-α concentrations were higher in both uncomplicated malaria and severe malarial anemia patients ($p < 0.001$) (Table 2).

sFasL concentrations

The mean sFasL serum concentrations in children with asymptomatic infections were very similar to those with uncomplicated malaria. The highest levels were detected in the group with severe malarial anemia ($p < 0.001$).

Plasma IL-10 to TNF-α ratio

The ratio of IL-10: TNF-α was not significantly different between the 3 groups.

Plasma IL-10 to sFasL ratio

The ratio of IL-10: sFasL was not significantly different between the 3 groups.

Considering all the children, we observed that the concentrations of hemoglobin were negatively correlated with the levels of TNF-α (rho = – 0.78, $p < 0.001$), sFasL (rho = – 0.63, $p < 0.001$) and IL-10 (rho = – 0.29, $p = 0.006$). No correlation between hemoglobin concentrations and ratio of IL-10: TNF-α (rho = 0.09, $p = 0.4$) and IL-10: sFasL (rho = 0.17, $p = 0.1$) was found.

In addition, the association between the parasite density and IL-10, TNF-α, and sFasL levels was assessed. We found a positive correlation with the levels of the 3 cytokines IL-10 (rho = 0.25, $p = 0.02$), TNF-α (rho = 0.69, $p < 0.001$) and sFasL (rho = 0.27, $p = 0.01$), but no association with IL-10: TNF-α and IL-10: sFasL ratios.

To investigate the effect of parasitemia and levels of IL-10, TNF-α and sFasL on hemoglobin concentrations, a multiple regression model was constructed. The variables sFasL ($p < 0.001$) and TNF-α ($p < 0.001$), but not IL-10 and parasitemia, strongly predicted low hemoglobin concentrations.

DISCUSSION

P. falciparum malaria is commonly associated with anemia in African children. In the present study, we investigated the association of the levels of IL-10, TNF-α and sFasL with hemoglobin concentrations in young children with *P. falciparum* infections living in Lambaréné where the most common complication of malaria is severe anemia.

The relative importance of acute, high parasite density compared with a persistent low-parasitemia in the development of malarial anemia remains unclear. It has been shown that anemia could also occur during asymptomatic malarial parasitemia [16]. In our study, we showed an association between the level of parasite density at admission and the concentration of hemoglobin in patients. Data obtained from Tanzania suggested that malaria-related anemia should be defined as a reduction in the hemoglobin level for the age and sex, in the presence of a malarial parasitemia of any density in endemic areas [2].

We found that serum TNF-α, IL-10 and sFasL levels were associated with the parasite density and the hemoglobin concentrations. During the acute phase of uncomplicated malaria, serum levels of IL-10 and sFasL were not affected, only the level of TNF-α was increased. All the cytokines levels evaluated here were increased in serum samples from children with severe malarial anemia, and this increase was particularly striking for sFasL concentrations. The apoptotic role of the Fas/FasL system has been shown in the regulation of erythropoiesis [17]. As has been reported for other diseases, the presence of high levels of sFasL may also play an important pathogenetic role in dyserythropoesis and anemia due to malaria. It was also found that, Fas-induced apoptosis was antagonized by high levels of erythropoietin [17].

We found a similar IL-10:TNF-α ratio in the asymptomatic and severe malarial anemia groups. This contrasts with results from a study carried out in Kenya [10], in which the mean IL-10:TNF-α ratio was significantly higher in children with mild and high-density parasitemia than in children with anemia. In other studies undertaken in Gabon [12] and Ghana [4], a significantly lower IL10:TNF-α ratio was found in plasma samples from patients with severe anemia. The discrepancy with our previous study carried out in Gabon could be due to the different age groups considered in these studies. However, we suggest that the ratio, by itself, does not predict malarial complications.

In conclusion, this study shows that the levels of TNF-α and sFasL are strongly and independently, negatively correlated with the hemoglobin concentration, and that the

sFasL is a better indicator for severe malarial anemia than for uncomplicated malaria.

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