MALARIA-ASSOCIATED CYTOKINE CHANGES IN THE PLACENTA OF WOMEN WITH PRE-TERM DELIVERIES IN YAOUNDE, CAMEROON

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Abstract. The prevalence of pre-term deliveries (PTDs) is increased in women who become infected with Plasmodium falciparum during pregnancy. Because prematurity is a risk factor for newborns, it is important to identify conditions that contribute to malaria-associated PTDs. Plasmodium falciparum–infected erythrocytes sequester in the placenta and attract activated mononuclear cells that secrete pro-inflammatory cytokines. Increased inflammatory cytokine levels in other microbial infections are associated with PTDs. To determine if such is the case in women with placental malaria, concentrations of interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin-4 (IL-4), and IL-10 were measured in placental plasma of 391 malaria-infected and -uninfected Cameroonian women with premature and full-term deliveries. Risk factors for malaria-associated PTDs included peripheral and placental parasitemias greater than 1%, maternal anemia, elevated IL-10 levels, and low TNF-α:IL-10 ratios due to over-expression of IL-10. Alterations in cytokine levels may contribute to PTDs through the induction of anemia and/or altering cellular immune responses required for eliminating placental parasites.

INTRODUCTION

Women who become infected with Plasmodium falciparum during pregnancy have an increased risk of delivering low birth weight babies, due either to pre-term delivery (PTD) or intra-uterine growth retardation.1–3 Malaria in pregnant women also contributes significantly to infant mortality. In sub-Saharan Africa, it is estimated that up to 200,000 infants die annually as a result of maternal infection during pregnancy.4 Accordingly, there is significant interest in understanding how malaria influences fetal development and pregnancy outcome.

PTD is a leading cause of neonatal mortality.5 Neurologic and respiratory systems of premature infants may not have completed development at birth, causing infants to have a variety of morbidities.6 Most premature infants are also low birth weight, thereby increasing their risk of dying during their first year of life.6 At least 40% of all pre-term births in developed countries occur in mothers with intrauterine infections, mainly of bacterial etiology.7 In tropical countries, malaria is a significant risk factor for premature deliveries.8–10

During successful pregnancies, fetal trophoblasts and maternal leukocytes secrete predominantly Th2-type cytokines, e.g., interleukin-10 (IL-10), to prevent initiation of inflammatory and cytolytic-type responses that might damage the integrity of the maternal-fetal placental barrier.11,12 In response to invading pathogens, however, Th1-type cytokines may be produced that reverse the Th2-type bias within the placenta. Excessive secretion of inflammatory cytokines against microbial pathogens along with insufficient quantities of counter-regulatory IL-10 are reported to be important factors associated with PTD.13,14 The sequestration of P. falciparum parasites in the intervillous space of the placenta also stimulates the production of Th1-type cytokines, including interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α).15–17 Early studies by Clark and Chaudhri demonstrated that elevated TNF-α levels in pregnant mice infected with P. vinckei result in fetal death and abortion.18 The influence of TNF-α and other pro-inflammatory cytokines on pregnancy outcomes in humans with malaria is less clear. Significant increases in TNF-α levels have been reported in pregnant women as a result of malaria, yet the majority of women had full-term deliveries (FTDs).15–17 Moorman and others found no association between increased TNF-α mRNA expression in the placenta and PTDs among malaria-infected women in Malawi.19 The investigators, however, noted that the sample size was small (n = 44, with eight cases of PTD), making the conclusion provisional. Therefore, the relationship between cytokine production at the maternal-fetal interface and pregnancy outcome, specifically PTD, requires elucidation.

The current study sought to determine whether malaria-related cytokine changes in the placenta of women with PTDs were significantly different from those in women who had FTDs. Based on previous studies, we hypothesized that in PTDs high placental parasitemias during the third trimester would lead to elevated levels of TNF-α without the production of compensatory levels of IL-10. Results from the current study showed that high placental parasitemia and maternal anemia were important risk factors for malaria-related PTD, but contrary to predictions, both TNF-α and IL-10 were significantly elevated in malaria-infected women with PTDs. An association between parasitemia, anemia, and IL-10 was found, suggesting that high IL-10 levels are associated with pathologies contributing to PTD.

MATERIALS AND METHODS

Subjects. The study was reviewed and approved by the Institutional Review Board of Georgetown University, the National Ethical Committee, Ministry of Health, Cameroon, and is covered by Single Project Assurance #S-9601-01. Women residing in the capital city of Yaounde and nearby surrounding areas who delivered at the Biyem Assi Hospital between August 1996 and August 1997 and Central Hospital from September 1997 to August 2001 were consecutively recruited. A member of the hospital staff explained the purpose

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of the study to each woman and, only after obtaining verbal informed consent, was the woman formally enrolled. A questionnaire was used to record relevant clinical information, including maternal age, gravidity, use of anti-malarial drugs during pregnancy, and date of last menstrual period. After delivery, information about the newborn was also recorded. Infants born between the 28th and 37th weeks of gestation were considered premature.

**Study design.** Women were excluded if they had abortions, stillbirths, caesarian sections, or multiple births, i.e., only women with singleton, vaginal deliveries were included. A detailed description of the 391 women included in the study is provided in Table 1. Among the women recruited, 83 women had PTDs. To compare women with PTDs with those who had FTDs, a representative group of 308 women with FTDs was selected by frequency matching with respect to placental malaria infection.

**Sample collection.** Immediately after delivery, ~5 mL of maternal placental blood was collected using the biopsy-pool method for cytokine analysis. Briefly, a block of tissue (5 cm x 5 cm x 5 cm) was excised from the basal side of the placenta, resulting in the formation of a large pool of intervillous blood at the excision site. Heparinized blood was quickly withdrawn and placed on ice until processed in the laboratory. A piece of placental tissue (2 cm x 2 cm x 2 cm) was also collected, and a portion was placed in 10% buffered formalin and processed for histology using routine methods. The remainder of the tissue was used to prepare impression smears for parasitologic analysis. In addition, ~5 mL of heparinized, maternal venous blood was collected for hematologic and parasitologic analysis.

**Parasitemias and anemia.** Thick and thin blood films were prepared using maternal peripheral and placental blood. Impression smears of placental tissue were also prepared. The slides were stained with Diff-Quick solution (Baxter Scientific Products, Miami, FL) and examined for the presence of *P. falciparum* parasites. Parasitemias were calculated by counting the number of infected erythrocytes per 2,000 erythrocytes and expressing the value as a percentage. Based on the presence/absence of parasites in placental impression smears, smear of placental blood, and histologic sections, women were classified as either positive or negative for placental malaria.

An aliquot of maternal peripheral blood was used to determine the packed cell volume (PCV). Using the definition of the World Health Organization, a woman was considered anemic if the PCV was less than 30%.

**Measuring cytokine levels in placental plasma by an enzyme-linked immunosorbent assay (ELISA).** Plasma samples collected prior to August 1997 (n = 175) were assayed for IFN-γ, TNF-α, IL-4, and IL-10 using the DuoSet ELISA Development System (Genzyme Diagnostics, Cambridge, MA), while samples collected thereafter were assayed for the same cytokines using ELISA kits from BD Pharmingen (San Diego, CA). The sensitivity of the assays was comparable: 2 pg/ml for IFN-γ and IL-10 and 5 pg/ml for TNF-α and IL-4.

**Statistical analyses.** The Wilcoxon rank sum test was used to determine significant differences in the amounts of cytokines and other continuous variables between malaria-infected and non-infected women who delivered prematurely or at term. The Pearson’s chi-square test was used for uni-

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**TABLE 1**

Comparison of women with pre-term deliveries and full-term deliveries

|                      | All women | Malaria + | Malaria - | P | All women | Malaria + | Malaria - | P | P +
|----------------------|-----------|-----------|-----------|---|-----------|-----------|-----------|---|---
| Number studied       | 83        | 38        | 45        |   | 308       | 110       | 198       |   |   |
| Maternal age (years) | 25.0 ± 6.4| 24.0 ± 5.0| 25.9 ± 7.4| 0.20| 24.8 ± 5.4| 24.5 ± 5.3| 24.9 ± 5.5| 0.45| 0.68|
| Primigravidae        | 41.5%     | 36.8%     | 44.4%     | 0.48| 39.9%     | 40.4%     | 39.1%     | 0.85| 0.81|
| Anti-malarial drug usage| 86.7%  | 84.2%     | 88.9%     | 0.53| 90.0%     | 90.9%     | 87.9%     | 0.10| 0.58|
| Placental parasitemias | (%)*     | 2.1%      | 0         |   | 0.7%      | 0         |   |   |   |
| Peripheral parasitemias (0%, 0.8%)* | 0.12%‡ | 0         | 0         |   | 0.03%     | 0         |   |   |   |
| Packed cell volume‡ | 31.8 ± 6.4%| 29.4 ± 5.9%| 34.4 ± 5.4%| <0.001| 34.4 ± 6.1%| 33.8 ± 6.0%| 34.8 ± 6.2%| 0.05| 0.003|
| % Anemia              | 46.7%     | 75.8%     | 23.8%     |   | 28.2%     | 33.3%     | 25.1%     | 0.12| 0.003|
| Gestational age (weeks)‡ | 33.1 ± 3.2| 33.4 ± 2.8| 32.9 ± 3.5| 0.47| 40.1 ± 1.7| 40.1 ± 1.6| 40.1 ± 1.8| 0.90| <0.0001|
| Infant birth weight (grams)‡ | 2,159 ± 651| 2,150 ± 607| 2,166 ± 693| 0.91| 3,322 ± 441| 3,235 ± 410| 3,370 ± 451| 0.01| <0.0001|
| IFN-γ (pg/mL)#       | 13.2 ± 10.4| 13.5 ± 10.5| 12.8 ± 10.6| 0.43| 13.9 ± 27.1| 20.4 ± 40.4| 9.5 ± 10.5| <0.0001| 0.07|
| TNF-α (pg/mL)‡       | 48.5 ± 105.2| 63.4 ± 124.6| 36.7 ± 86.6| 0.012| 45.7 ± 107.6| 43.9 ± 99.3| 45.8 ± 111.1| 0.61| 0.17|
| IL-4 (pg/mL)‡        | 9.5 ± 6.4| 8.6 ± 6.2| 10.5 ± 6.6| 0.19| 12.8 ± 29.3| 15.6 ± 43.1| 11.0 ± 15.5| 0.23| 0.43|
| IL-10 (pg/mL)‡       | 229.5 ± 556.3| 449.1 ± 764.2| 42.8 ± 85.1| <0.0001| 51.9 ± 92.5| 72.5 ± 107.0| 37.1 ± 77.5| 0.003| 0.0003|

* IFN = interferon; TNF = tumor necrosis factor; IL = interleukin. Bold numbers indicate statistical significance.
† Comparison between all women with PTD and FTD.
‡ Mean ± SD.
§ Median (25th percentile, 75th percentile).
¶ In this study, 26.5% of women with placental malaria were peripheral blood smear negative, consistent with a previous report.
# Mean ± SD (median).
Placental malaria in women with PTDs did not have a significant impact on either infant birth weight ($P = 0.91$) or mean gestational age ($P = 0.47$) (Table 1). However, placental malaria was associated with a reduction in infant birth weight among women with FTDs ($P = 0.01$) (Table 1).

**Cytokine levels in placental plasma.** The distribution of IFN-γ, TNF-α, IL-4, and IL-10 in placental plasma of women with PTDs and FTDs is shown in Figure 2. No significant differences in cytokine levels were observed between malaria-negative women with PTDs and FTDs for the four cytokines ($P > 0.05$ in all comparisons). Thus, PTD, by itself, was not associated with an alteration in the placental cytokine balance.

Conversely, both TNF-α and IL-10 were significantly elevated in malaria-positive women with PTDs compared with women with FTDs (Figure 2). The median level of TNF-α in malaria-positive women with PTDs was more than twice that found in infected women with FTDs ($P = 0.012$). In contrast, the median IL-10 level of malaria-infected women with PTDs was four-fold higher than that in infected women with FTDs ($P < 0.0001$) (Table 1 and Figure 2). These data support the hypothesis that TNF-α levels are elevated in malaria-infected women with PTDs, but reject the hypothesis that IL-10 synthesis is suppressed. In fact, infected women with PTDs had very high levels of IL-10 (Figure 2). Although IL-10 levels were also elevated 1.5-fold in infected women with FTDs (27.8 pg/mL) compared with non-infected women (18.0 pg/mL; $P = 0.003$), the increase was significantly less than that observed in PTDs (Table 1 and Figure 2). However, a significant correlation between TNF-α and IL-10 levels exists within the same individual of all infected women (Spearman’s $\rho = 0.62, P < 0.0001$).

Since both TNF-α and IL-10 were significantly elevated in malaria-infected women with PTDs, the ratio of TNF-α to IL-10 for each woman in the study was determined and TNF-α:IL-10 ratios were compared. No significant differences were found in median values for malaria-negative women with FTDs and PTDs (median = 0.59 versus 0.53, respectively; $P = 0.94$) or malaria-positive women with FTDs (median = 0.49). However, significantly lower TNF-α:IL-10 ratios were found in malaria-positive women with PTDs compared with those with FTDs (median = 0.28 versus 0.49, respectively; $P = 0.004$). Thus, TNF-α:IL-10 ratios were similar in women with successful FTDs and in malaria-negative women with PTDs, but they were significantly lower in women with PTDs with placental malaria.

**Risk factors associated with PTDs in malaria-positive women.** Our results show that malaria-infected women with PTDs had higher parasitemias, (Table 1 and Figure 1), lower PCV values (Table 1), higher levels of placental TNF-α and IL-10 (Table 1 and Figure 2), and lower TNF-α:IL-10 ratios. Therefore, risk factors for PTD were assessed by calculating the odds ratios associated with these variables (Table 2). Univariate analysis showed that both peripheral and placental parasitemias greater than 1%, maternal anemia, elevated levels of IL-10, and low TNF-α:IL-10 cytokine ratios were all significant risk factors for PTDs (Table 2). After adjusting for other covariates, only maternal anemia remained significantly associated with PTDs ($P = 0.005$). Significant correlations between IL-10 and placental parasitemia (Spearman’s $\rho = 0.38, P < 0.0001$) and between TNF-α and placental parasitemia (Spearman’s $\rho = 0.10, P = 0.02$) were also found.
When women were categorized based on their placental parasitemias and stratified according to anemia, anemia did not appear to enhance the prevalence of PTD among malaria-negative women (Figure 3). Among malaria-infected women with low placental parasitemias (< 1.0%), the proportion of women who delivered prematurely was significantly higher in the anemic compared with the non-anemic group (24.1% versus 7.1%, respectively; \( P = 0.045 \)). However, the most dramatic increase in the proportion of women with PTDs was observed among women with high placental parasitemias (> 1.0%) who were also anemic (i.e., 60.7% of women in this group have PTDs), which represents a 3.6-fold increase compared with the proportion of women with PTDs in non-anemic group with similar levels of placental parasitemias (18.5%; \( P < 0.001 \)) (Figure 3).

Since anemia was a significant risk factor for PTDs, risk factors for maternal anemia were also evaluated. Univariate analyses showed that risk factors for maternal anemia included placental parasitemias > 0.1% and elevated levels of IL-10 (Table 3). After adjusting for other covariates, only placental parasitemias > 0.1% was significantly associated with anemia. Because cytokines have been implicated in the development of anemia\(^{20,21}\) and high parasite densities,\(^{22,23}\) correlations between 1) TNF-\( \alpha \) and PCV, 2) IL-10 and PCV, 3) TNF-\( \alpha \) and placental parasitemia, and 4) IL-10 and placental parasitemia were determined. A significant negative correlation was found between IL-10 and PCV (Spearman’s \( r = -0.20, P = 0.002 \)), while there was no correlation between TNF-\( \alpha \) and PCV (Spearman’s \( r = -0.004, P = 0.94 \)). Taken together, these results suggest that among malaria-infected women, high placental parasitemias and maternal anemia dramatically increase the risk of PTD and that these risk factors are associated with elevated IL-10 levels.

**DISCUSSION**

The identification of factors contributing to malaria-associated PTD are extremely important because low birth weight babies born prematurely have an increased risk of dying during the first year of life. Pregnant women who become infected with bacteria often produce pro-inflammatory cytokines including TNF-\( \alpha \).\(^{24}\) Production of TNF-\( \alpha \), in the absence of compensatory production of IL-10, has been associated with spontaneous abortions and PTDs.\(^{14,25}\) Since *P. falciparum* parasites sequester in the intervillous space and stimulate the infiltration of monocytes/macrophages\(^{26}\) that secrete TNF-\( \alpha \), it was logical to speculate that increased levels of placental TNF-\( \alpha \) and low levels of IL-10 would be contrib-
uting factors to PTDs in malaria-infected women. Results from the present study suggest that this scenario is not correct. That is, women with placental malaria who have PTDs produce significantly higher amounts of IL-10 than women with FTDs (Figure 2), and their placental TNF-α/IL-10 ratio was significantly biased towards a Th2-profile. Thus, inadequate IL-10 production does not appear to be a contributory factor to PTD in the context of placental malaria. Since a significant correlation between TNF-α and IL-10 (within the same individual) was found in women with placental malaria, it is likely that a feedback mechanism has been induced to help regulate the inflammatory process at the fetal-maternal interface.

The present study identified several risk factors for malaria-related PTD after adjusting for age and gravidity (Table 2). The major risk was having a placental parasitemia of greater than 1% at the time of delivery, confirming reports by other investigators that high placental parasitemias significantly influences PTD.\(^8\) In addition, anemia, which is one of the most common complications of malaria during pregnancy, especially in primigravidae,\(^27,28\) was also identified as a risk factor for PTD (Table 2). This combination of factors is an important predictor for PTD (Figure 3). Since parasite densities and the development of anemia are influenced by cytokines,\(^20–23\) it was not surprising that changes in cytokine levels were also found to be risk factors for PTD. These included a low placental TNF-α/IL-10 ratio due to excessive production of IL-10, and high placental IL-10 levels alone (Table 2).

The immunologic relevance of the ratio of TNF-α and IL-10 concentrations found in peripheral plasma samples remains controversial in the literature. Several studies have reported that high TNF-α and low IL-10 levels are associated with severe malaria in children,\(^29–31\) whereas other studies have found an association between elevated levels of IL-10 and high parasitemias.\(^22,23,32,33\) Studies in murine models of malaria have also suggested that IL-10 response during infection may be associated with disease exacerbation.\(^34\) It is be-
believed that high IL-10 levels impair the inflammatory response required for parasite killing, resulting in higher parasitemias. However, information on the balance between TNF-α and IL-10 and its role in malaria protection/pathogenesis remains to be elucidated.

In the present study, *P. falciparum*-infected women with PTDs had elevated levels of both TNF-α and IL-10 (Figure 2), but the TNF-α:IL-10 ratio was significantly decreased, suggesting a Th2-biased response. During pregnancy, the overall immune response of the mother is Th-2 biased to prevent fetal allograft rejection. While both maternal leukocytes and fetal trophoblasts are capable of producing IL-10 during *P. falciparum* infection, maternal monocytes and macrophages are the most likely source of placental IL-10 in malaria-infected women. Massive monocytic infiltration is a hallmark of placental malaria, and increased migration of monocytes and macrophages to the placental intervillous space has been identified as a risk factor for PTD. In women with malaria-associated PTDs, it is possible that elevated levels of IL-10 suppress anti-parasite inflammatory responses resulting in high placental parasitemias and ultimately anemia. In this study, a significant correlation was found between IL-10 and placental parasitemia, as well as a negative correlation between IL-10 levels and PCV. In addition, a significant correlation was also found between TNF-α and placental parasitemia but not with PCV. The latter result was somewhat surprising given that the role of TNF-α in the development of anemia in the context of malaria is well established. However, recent evidence suggest that both pro- and anti-inflammatory cytokines are involved in the pathogenesis of anemia of chronic diseases. Elevated levels of IL-10 can increase the acquisition and retention of iron by monocytes and macrophages and increase ferritin synthesis, thereby reducing the amount of iron in the plasma and thus contributing to anemia. Since many cases of placental malaria are chronic in nature and anemia places a stress on iron metabolism, it is probable that the elevated levels of IL-10 contribute significantly to the development of maternal anemia, a known risk factor for PTD. Human IL-10 has also been shown to suppress the production of granulocyte-macrophage colony-stimulating factor by T cells resulting in the inhibition of erythroid progenitor cells, presenting another mechanism on how IL-10 may contribute to anemia. Thus, both elevated levels of pro-inflammatory TNF-α and regulatory IL-10 may contribute to anemia in pregnant women.

Information from the present study should be applicable to women living in urban cities throughout Cameroon and other African countries where malarial transmission is perennial. The pregnant women in the study represented a diverse group of women, with different ages (15–50 years old), numbers of pregnancies (1–11), and a range of economic and social backgrounds (Table 1). Their malariometric profile, however, is similar to that reported for pregnant women in other African countries. The influence of other diseases on the results is difficult to assess, including infection with human immunodeficiency virus, which infects an estimated 4.0–13.6% of women attending antenatal clinics in urban areas of Cameroon in 2001. However, because all consecutively recruited women with PTDs were studied and women with FTDs were randomly selected, it is unlikely other diseases had a major impact on the results. There is no reason to suspect that placental malaria was not the key factor influencing the cytokine changes observed in this study (Figure 2).

In summary, results from this study are consistent with the following scenario. In pregnant women, malarial parasites sequester in the placenta and stimulate the accumulation of activated macrophages in the intervillous space where they secrete large amounts of TNF-α. In response to this inflammatory challenge, maternal and fetal cells secrete IL-10 to limit pathology and to protect the fetal allograft. Although IL-10 is important in modulating the possible deleterious effects of inflammatory cytokines, its over-expression may have
detrimental consequences resulting in PTD by enhancing maternal anemia and suppressing anti-parasite inflammatory responses leading to persistent placental parasitemias.

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