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Placental Tumor Necrosis Factor Alpha but Not Gamma Interferon Is Associated with Placental Malaria and Low Birth Weight in Malawian Women

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Malaria in pregnancy predisposes to maternal anemia and low birth weight (LBW). We examined the possible roles of the cytokines tumor necrosis factor alpha (TNF-α) and gamma interferon (IFN-γ) in these adverse outcomes. We measured cytokine concentrations in placental, peripheral, and cord blood plasma in relation to malaria parasitemia and placental monocyte accumulation in 276 Malawian women. Maternal hemoglobin concentration, human immunodeficiency virus status, and infant birth weight were determined. Concentrations of TNF-α in placental blood were correlated with densities of *Plasmodium falciparum*-infected erythrocytes ($P < 0.0001$) and of intervillous monocyte infiltrates ($P < 0.0001$) on placental histology. Peripheral blood TNF-α concentrations were relatively low and were weakly associated with malaria. TNF-α concentrations were higher in placental blood, where they were strongly associated with malaria. Placental plasma TNF-α levels were higher in women who had LBW babies ($P = 0.0027$), women with febrile symptoms ($P < 0.0001$), and teenage mothers ($P = 0.04$) than in other women. The presence of TNF-α in cord blood was not associated with malaria infection. IFN-γ levels were infrequently elevated, and elevated IFN-γ levels were not associated with poor pregnancy outcomes. Placental production of TNF-α, but not of IFN-γ, may be implicated in impaired fetal growth in Malawian women.

Malaria in pregnancy can have adverse consequences for both mother and infant. Malaria increases the risk of anemia in pregnant women and is the single most important preventable cause of low birth weight (LBW), which is associated with high infant mortality (28). The mechanisms by which malaria exerts these adverse effects are poorly understood. Placental infection with *Plasmodium falciparum* may be accompanied by mononuclear cell infiltrates (3, 30). Dense monocytic infiltrates within the intervillous space are not infrequent in malaria infection and are rare in the absence of malaria (19). These infiltrates are associated with intrauterine growth retardation in malaria-exposed pregnancies (12). Recently, we have found that the presence of malaria pigment-containing fibrin or leukocytes and the numbers of placental monocytes independently predict low infant birth weight and low maternal hemoglobin (23a).

In normal human pregnancy, a placental bias toward T helper 2 (Th2) responses (13) may be important for successful pregnancy (21). In placental malaria infection, a proinflammatory or Th1 cytokine response appears common. Cytokines associated with placental malaria have been measured by a number of techniques, including assays of placental serum (8, 9), RNase protection assay (18), and secretion in vitro by placental blood cells, purified mononuclear cells, or villous explants (5, 8, 16, 17). Malaria-associated increases in tumor necrosis factor alpha (TNF-α), interleukin 8 (IL-8), gamma interferon (IFN-γ), IL-6, and IL-10 have been reported in one or more assay systems, but only increased placental TNF-α levels have been repeatedly associated with LBW (9, 18).

To further investigate associations between malaria, maternal anemia, and LBW, we have quantified leukocytes and parasitized erythrocytes within the placental intervillous space and measured concentrations of the cytokines TNF-α and IFN-γ in placental, peripheral, and cord plasma. We have examined the influence of human immunodeficiency virus (HIV) infection in this process and used multivariate analysis to determine whether TNF-α concentrations are associated with poor pregnancy outcomes, independently of placental parasitemia.

MATERIALS AND METHODS

Women attending Queen Elizabeth Central Hospital, Blantyre, Malawi, for delivery were screened for malaria infection by examination of thick films from placental and peripheral blood. Giemsa-stained films were examined by counting parasites against 200 leukocytes, and parasitemia was determined by using an assumed leukocyte count of 6,000/μl. Women with malaria infection (peripheral and/or placental parasitemia of >1,000/μl on thick-film microscopy) and uninfected controls (matched for gravidity and for age within ±2 years, but with peripheral and placental thick blood films negative for malaria) were recruited into the study following witnessed informed consent. A placental parasite density of ≥1,000/μl was seen in 77.5% of infected women in this study, but in only 48.9% of infected women delivering in the hospital (S. J. Rogerson, E. Chaluluka, L. Njiragoma, M. Kanjala, P. Mkundika, and M. E. Molyneux, unpublished data).

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Blood films were examined by a second microscopist, with a third read to resolve conflicting results, as described elsewhere (23). Final parasitemia was the average of two agreeing counts. Babies were weighed at delivery; LBW was defined as <2,500 g.

Venous blood was collected from a peripheral vein and cord blood was collected from umbilical veins by venipuncture. Placental blood was collected by incising the maternal surface of the placenta (which was cleaned with sterile gauze and normal saline) and aspirating blood welling into the incision with a sterile transfer pipette. Heparinized (first 40 cases) or EDTA plasma was separated within an hour of delivery and frozen at −70°C until it was aliquoted for assay. Placental tissue biopsy specimens (2 by 2 by 1 cm) were collected into neutral buffered formalin. After fixation, blocks were wax embedded, and sections stained with Giemsa stain or hematoxylin and eosin were prepared by standard procedures. Maternal hemoglobin concentrations were measured with a Hemocue (Angelholm, Sweden) hemoglobinometer.

Placental tissue examination. Placental sections were examined by a standardized approach, and parasite and monocyte densities were determined by counting 500 intervillous-space cells, including infected and uninfected erythrocytes and leukocytes (23a). Parasitemia was expressed as the number of infected erythrocytes per total number of erythrocytes. Monocyte density was calculated as the number of monocytes detected per total erythrocytes and leukocytes counted. Each was expressed as a percentage. Malaria pigment (hemozoin) in monocytes and in fibrin was assessed by using a semiquantitative scale (score, 0 to 4) and recorded as present or absent.

HIV testing. For the first 120 samples, HIV testing was performed on coded samples, without patient identifiers. For subsequent samples, HIV testing followed voluntary counselling and testing, performed the day after delivery. Plasma or serum was tested by a Serocard rapid test for HIV types 1 and 2 (HIV-1 and -2) (Trinity Biotech, Dublin, Ireland), and results were confirmed by either HIV-1 and -2 ELISA (Ortho-Clinical Diagnostics, Neckargemund, Germany), Vironostika HIV Uni-Form II (Organon Teknika, Boxtel, The Netherlands), or Determine (Abbott Laboratories, Amadora, Portugal).

Cytokines. Cytokine assays were performed on consecutive placental plasma samples available from women enrolled in the study. Placental and maternal or cord blood cytokine concentrations were measured for TNF-α and IFN-γ by using enzyme-linked immunosorbent assay kits from R&D Systems, Abingdon, United Kingdom, according to the manufacturer's instructions. Additional cord blood samples were assayed for TNF-α by using the human TNF-α Duoset (R&D Systems, Minneapolis, Minn.). Only samples collected into EDTA were analyzed for IFN-γ. Limited peripheral blood samples were assayed owing to lack of reagents. For TNF-α, assays were performed on peripheral, placental, and cord blood samples from 20, 241, and 141 individuals, respectively. For IFN-γ the numbers were 32, 187, and 53 samples. From information supplied by the manufacturer, the upper limits of normal were defined as 15.6 pg/ml for both IFN-γ and TNF-α.

Statistical analysis. Data were entered into Microsoft Access and transferred to Stata 6.0 (Stata Corp., College Station, Tex.) for analysis. Normally distributed data were compared by Student's *t* test, and nonnormally distributed data were compared by the Wilcoxon rank sum test.

Ethical approval. The studies were approved by the College of Medicine Research Committee, University of Malawi.

RESULTS

Of 276 women in the study, placental histology was available for 254. By histology, 102 (40%) were uninfected; of these, 3 had parasitemia on placental film only and 2 had parasitemia on maternal film only. One hundred fifty-two women (60%) had parasites on placental histology. Of these, 85 were positive on peripheral and placental blood films, 14 were positive on placental but not maternal blood film, and 53 were negative on both blood films. A further 43 women (17%) had fibrin pigment but no parasites on histology, indicative of previous infection.

Fifty-eight percent of women with malaria had experienced febrile symptoms in the previous week, compared to 18.8% of women without malaria (odds ratio [OR], 6.1; 95% confidence interval [95% CI], 3.3 to 11.1; $P < 0.001$). Birth weights and maternal hemoglobin concentrations were compared between women with and without malaria on peripheral or placental blood film or placental histology. Birth weights differed most significantly between women with and without peripheral parasitemia (2.65 ± 0.51 kg compared to 2.90 ± 0.47 kg; $P = 0.0001$) and least between women with and without malaria on placental histology (2.77 ± 0.50 kg compared to 2.89 ± 0.52 kg; $P = 0.056$). Mean hemoglobin concentrations were 11.1 ± 1.8 g/dl for women with malaria on histology and 12.0 ± 1.9 g/dl for women without active infection ($P = 0.0003$). Differences in hemoglobin levels were similar however malaria was defined ($P < 0.002$ for each comparison).

TABLE 1. TNF-α concentrations in placental, peripheral, and cord blood in relation to malaria infection of the placenta

Blood	Median TNF-α concn (interquartile range); no. of samples tested		*P* (rank sum)
	Malaria	No malaria	
Placental	10.0 (4.0–24.0); 142	3.3 (1.0–8.3); 99	<0.0001
Peripheral	2.9 (1.63–6.4); 14	0.47 (0.18–0.96); 8	0.026
Cord	7.9 (0–19.0); 86	2.5 (0–16.2); 55	0.87

Levels of TNF-α were higher in placental than in peripheral or cord blood and were higher in infected than in uninfected placentas (Table 1). Differences in placental ($P < 0.0001$) and peripheral ($P < 0.05$) blood levels were similar if malaria was categorized on the basis of peripheral or placental parasitemia rather than histology. High concentrations of TNF-α in placental plasma (>15.6 pg/ml) were detected in 66 women with placental histology available, 53 of whom (80.3%) had malaria infection (OR, 3.9; 95% CI, 2.0 to 7.7; $P < 0.001$). Placental TNF-α levels were correlated with placental parasitemia ($r = 0.40$; $P < 0.0001$) and density of placental monocytic infiltrates ($r = 0.39$; $P < 0.0001$). Placental TNF-α concentrations were significantly higher in LBW babies (median, 11.6 pg/ml) than in babies weighing ≥2,500g (median, 5.6 pg/ml; $P = 0.0045$) (Table 2). The relationship between malaria and TNF-α was independent of gravidity. TNF-α levels correlated with placental parasitemia in primigravidae ($r^2 = 0.42$; $P < 0.0001$) and women in their second or later pregnancy ($r^2 = 0.35$; $P = 0.0001$). After stepwise regression, placental parasitemia on histology ($P = 0.031$), gravidity ($P = 0.003$), and maternal hemoglobin ($P = 0.007$) were significantly associated with infant birth weight, whereas TNF-α was not ($P = 0.26$).

Cord blood TNF-α concentrations were not associated with the presence or density of placental malaria infection (Table 1) or with infant birth weight ($r^2 = 0.04$; $P = 0.59$). Placental and cord blood TNF-α concentrations were not correlated ($r^2 = -0.03$; $P = 0.74$). Cord blood parasitemia was infrequent and low density (2.9%; maximum density, 150/μl). We did not have gestational assessments or postdelivery information on these babies.

IFN-γ levels were low in placental plasma but were slightly higher in 114 women with placental malaria (median, 0.7 pg/ml; interquartile range, 0 to 6.1 pg/ml) than in 73 uninfected women (median, 0; interquartile range, 0 to 0.57 pg/ml; $P = 0.0001$). IFN-γ levels were increased in only eight placental samples (4%), five with and three without malaria. IFN-γ was not detectable in 24 of 32 peripheral blood and 51 of 53 cord blood samples and was never elevated in these samples. IFN-γ levels were significantly higher in HIV-infected than in uninfected women without placental malaria (0.7 pg/ml [interquar-

TABLE 2. Patient characteristics and placental TNF-α concentrations

Patient characteristic[a]	Median placental TNF-α concn (interquartile range); no. of samples		P^b
	Factor present	Factor absent	
Teenager	8.2 (3.1–18.6); 94	5.6 (2.1–14.6); 166	0.04
Primigravid	6.9 (2.2–18.2); 136	5.6 (2.7–12.3); 123	0.27
Recent febrile symptoms	11.0 (4.6–26.0); 104	4.0 (1.7–9.7); 147	<0.0001
Anemic (Hb, <11 g/dl)	7.3 (2.9–19.6); 86	6.1 (2.7–15.8); 156	0.26
HIV infected	7.1 (2.8–18.6); 105	5.3 (2.1–14.2); 149	0.20
LBW	11.6 (4.6–24.9); 48	5.5 (2.1–12.3); 207	0.0045

[a] Hb, hemoglobin.
[b] Compared by the Wilcoxon rank sum test.

tile range, 0 to 6.1 pg/ml] compared to 0 [interquartile range, 0 to 0.6 pg/ml]; $P = 0.025$).

Table 2 compares placental TNF-α concentrations according to maternal characteristics and LBW. Young maternal age, recent febrile symptoms, and LBW infants were associated with increased placental TNF-α levels. Women with febrile symptoms, but without malaria, did not have increased placental TNF-α concentrations. HIV infection was not associated with TNF-α concentrations, nor was peripheral, placental, or histological parasitemia higher in HIV-infected than in uninfected women ($P > 0.38$ for all comparisons).

DISCUSSION

TNF-α is expressed in normal placentas by trophoblast cells and by cells thought to be macrophages (22), and it appears to play a role in term and preterm labor (reviewed in reference 1). Placental overproduction of TNF-α and other proinflammatory cytokines may be important in the development of preeclampsia, possibly in response to hypoxia (6), and has been implicated in the pathogenesis of intrauterine growth retardation (11), possibly by decreasing amino acid uptake by the fetus (4). Lipopolysaccharide may stimulate TNF-α production, leading to abortion in mice (26). Malarial glycosylphosphatidylinositol has lipopolysaccharide-like effects (25) and stimulates monocyte TNF-α release.

We measured placental, peripheral, and cord plasma TNF-α and IFN-γ levels and related them to the densities of placental malaria infection and monocyte infiltrates and to maternal hemoglobin and infant birth weight. Malaria-associated increases were largely confined to the placenta. Placental TNF-α levels were more commonly elevated in women who had placental malaria, who had recent febrile symptoms, or who gave birth to LBW babies. IFN-γ was usually detected only in low concentrations and was not associated with malaria or adverse pregnancy outcomes. Moormann et al. (18) found increased mRNA expression of TNF-α in placental tissues of Malawian women, findings similar to the changes in placental plasma protein levels that we detected. Our data confirm that the placenta is the likely site for production of TNF-α. Similar findings have been reported for women from Kenya and Cameroon (8, 9). In each case, levels of TNF-α were higher than those we detected, and serum (not plasma) was used for measurements.

Placental parasite densities were similar in women of different gravidity, and the placental TNF-α response to malaria did not differ according to gravidity. Gravidity-based differences in cytokine responses to malaria have been proposed (16) to explain the difference in susceptibility to malaria between primigravid and multigravid women (27), but our data do not support such a phenomenon in this population. Instead, humoral immune responses may be more important (2).

In malaria-infected placentas, TNF-α is produced principally by monocytes and macrophages (18), and intervillous monocyte infiltrates are associated with LBW in multivariate analyses (15, 23a). Placental malaria may exert some of its adverse effects on mother and child by triggering monocytic infiltration and local TNF-α production. Multivariate analysis (not previously applied to studies of cytokines in placental malaria) did not confirm an independent role for TNF-α in the pathogenesis of LBW. The short half-life of cytokines compared to the slow pace of changes in hemoglobin concentration or malaria parasitemia may militate against such findings. Alternatively, increased TNF-α levels may be a marker, rather than a mediator, of LBW. Only animal studies can resolve this issue.

Cord TNF-α concentrations were not associated with malaria infection at delivery. Increased cord TNF-α concentrations have been found in association with neonatal sepsis (24) and in premature infants were associated with cerebral lesions on magnetic resonance imaging (7). While some infants had high cord TNF-α concentrations, our study suggests that LBW associated with malaria is more likely due to changes such as TNF-α production at the level of the placenta. Placental and cord TNF-α levels were not associated, suggesting that passage of cytokines into cord blood is not a likely mechanism of TNF-α action.

We found strong associations between monocyte densities, malaria infection, febrile symptoms, and TNF-α concentrations, even though we were not able to exclude other infections, and we measured placental monocyte counts and cytokine concentrations only at delivery (Table 2). Women with fever and without placental malaria infection did not have elevated placental cytokine concentrations. Maternal febrile symptoms were associated with the presence of malaria, parasite densities, and cytokine levels (Table 2), suggesting that malaria infection caused a significant component of the febrile symptoms in this group.

Women with low-density infections had neither poor outcomes nor placental monocyte infiltrates, and they had lower cytokine levels than women with denser infections. Women in the more heavily infected group were at significantly higher risk of adverse pregnancy outcomes. There may be a threshold of infection density above which monocyte infiltrates and inflammatory responses occur.

Differences in placental IFN-γ responses to malaria infec-

tion between different African regions are intriguing. Two groups in Kenya have reported roles for IFN-γ in malaria-exposed pregnancies. Detectable IFN-γ levels (≥10 pg/ml) were found in about 40% of placental plasma samples from Kenyan women and were associated in certain patient groups with malaria infection or LBW (9). IFN-γ production by intervillous blood cells was associated with protection from malaria in pregnancy in multigravid women (16), and impaired IFN-γ production was mooted as a cause for the increased susceptibility of HIV-infected women to placental malaria (17). We found that although malaria-infected placentas had higher IFN-γ levels than did uninfected placentas, these levels were generally extremely low. HIV infection was associated with higher IFN-γ levels among women without malaria, consistent with the placental immune activation reported in HIV infection (14). Moorman et al. found no detectable IFN-γ or IL-12 mRNA in placental biopsy specimens from primigravid Malawian women (18), while IL-12 production was frequent in peripheral blood mononuclear cells from non-HIV-infected Kenyan women (5). IL-12 plays a key role in induction of IFN-γ production and appears to protect against malaria infection and anemia in mice (29). Differences in host response to placental malaria may have a genetic basis (20), as has recently been proposed for severe anemia (10).

Increases in placental TNF-α levels are associated with placental malaria and LBW. Whether cytokine production causes impaired fetal growth or is a marker of other processes resulting in poor pregnancy outcome requires further study.

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