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PLACENTAL MONOCYTE INFILTRATES IN RESPONSE TO *PLASMODIUM FALCIPARUM* MALARIA INFECTION AND THEIR ASSOCIATION WITH ADVERSE PREGNANCY OUTCOMES

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Abstract. Maternal anemia and low birth weight (LBW) may complicate malaria in pregnancy, and placental monocyte infiltrates have been associated with LBW, and anecdotally with anemia. We examined placental pathology from 357 Malawian women. Intervillous monocyte infiltrates were frequent in placental malaria and were not seen in uninfected placentas. Histology was grouped according to a 5-point scale. Dense monocyte infiltrates and presence of intramonocytic malaria pigment were associated with anemia and LBW. Of factors associated with LBW and/or anemia in univariate analysis, gravidity ($P = 0.002$), number of antenatal clinic (ANC) visits ($P < 0.001$), malaria pigment in fibrin ($P = 0.03$), and monocyte malaria pigment ($P = 0.0001$) remained associated with lower birth weight by multivariate analysis. Associated with maternal anemia were HIV infection ($P < 0.0001$), intervillous monocyte numbers ($P < 0.0001$), number of ANC visits ($P = 0.002$), and recent febrile symptoms ($P = 0.0001$). Pigment-containing placental monocytes are associated with anemia and LBW due to malaria, and may have a causative role in their development.

INTRODUCTION

Plasmodium falciparum malaria is more frequent in pregnant than non-pregnant women and increases the likelihood of maternal anemia[1] and low birth weight (LBW), predisposing to maternal and infant mortality.[2] Monocytes and macrophages are commonly seen in the placental intervillous spaces in malaria infection, and frequently contain malaria pigment.[3] Malaria pigment also is seen in fibrin deposits in the intervillous space (where it persists for an unknown period after parasites and monocytes clear).[3] Dense ("massive") intervillous monocyte infiltration was detected in placentas of 6.3% of Tanzanian women, and was independently associated with LBW, primarily due to intrauterine growth retardation (IUGR).[4,5] In small, observational studies, placental monocytes containing malaria pigment have been associated with decreased birth weight in first-born children[6] and with severe maternal anemia.[7]

Malaria-associated placental pathology has been classified according to presence or absence of parasites as well as presence or absence of malaria pigment (in fibrin or monocytes).[8–10] Bulmer et al. and Ismail et al. did not report differences in birth weight according to infection classification, while Leopardi et al. found that women with present, but not past, infection had significantly lower birth weight babies than uninfected women did.[10] Only infected placentas had increased leukocyte infiltrates, and there was a non-significant trend for birth weight to fall as infiltrate density increased. Mononuclear cells, which predominated in more-severe infections, were not differentiated into lymphocytes and monocyte/macrophages.[10]

To further explore whether placental monocyte-macrophage infiltrates (referred to subsequently as "monocytes") would be associated with maternal anemia and LBW, we developed methods for quantitating leukocytes and parasitized erythrocytes within the intervillous space, and for classifying placental histology. We took into account possible separate roles for monocytes and fibrin containing malaria pigment in the pathogenesis of the disease. We then prospectively examined the ability of these methods, together with the presence of malaria pigment in monocytes, to identify groups of women with high prevalence of maternal anemia and of LBW, and to identify independent risk factors for decreased birth weight and maternal hemoglobin concentration.

MATERIALS AND METHODS

Sample collection. Peripheral and placental blood of pregnant women at Queen Elizabeth Central Hospital in Blantyre, Malawi, for delivery were examined for malaria by microscopy of Giemsa-stained thick blood films, as described elsewhere.[11] Clinical information was obtained using questionnaires, and information on antenatal clinic (ANC) care from patient-held ANC cards. Number of doses of sulphadoxine pyrimethamine prescribed at the ANC were recorded. To divide antenatal visits roughly in half, we compared women with < 5 visits with those with ≥ 5. From January 1998 to March 1999, women with *P. falciparum* parasitemia > 1,000/μL in placental and/or maternal blood, together with age- and gravidity-matched controls, were recruited, and placental biopsies collected. A histology classification scheme was developed using this group of 117 women. From March 1999 to November 2000, we enrolled 357 women prospectively, irrespective of parasite count, if they delivered live-born infants while the study team was in attendance. Infants were weighed at delivery, and birth weight < 2,500 g was defined as LBW. Maternal peripheral and cord blood was collected within an hour of delivery, and full thickness placental biopsies obtained from a healthy pericentric area were placed into 10% neutral buffered formalin at delivery.

Placental examination and development of classification system. Fixed placental biopsies were wax embedded, and sections were stained with Gurr's modified Giemsa and/or hematoxylin and eosin. Slides were examined by either one

observer (E.P., pilot study) or two observers (S.J.R and A.G., main study), blinded to other patient data. Presence of *P. falciparum*-infected erythrocytes and of malaria pigment in fibrin and monocytes was noted. Using a systematic method, 500 intervillous blood cells were counted under oil immersion, and classified as uninfected erythrocytes (UE), infected erythrocytes (IE), or leukocytes, subdivided by morphology into lymphocytes, polymorphs, or monocyte-macrophages. Histologic percentage parasite density was defined as IE/(UE + IE), × 100%. Of leukocyte types, only monocyte counts varied between infected and uninfected placentas. Placental histology was classified according to the 5-point scale described in Table 1.

HIV testing. HIV testing followed voluntary counseling of women, performed after delivery. Plasma was tested by Serocard rapid test for HIV-1 and 2 (Trinity Biotech, Dublin, Ireland) and confirmed by HIV-1 and 2 enzyme-linked immunosorbent assay (Ortho-Clinical Diagnostics, Neckargemund, Germany), Vironostika HIV Uni-Form II (Organon Teknika, Boxtel, The Netherlands), or Determine (Abbott Laboratories, Amadora Portugal).

Ethical considerations. Written informed consent was obtained from all participants, and the study was approved by the College of Medicine Research Committee, University of Malawi.

Statistical analysis. Data were entered into Microsoft Access and transferred to Stata 6.0 (Stata Corp, College Station, TX) for analysis. For univariate analyses, normally distributed data were compared by Student's *t* test, and non-normally distributed data by Wilcoxon rank sum test. All factors reported in univariate analysis were included in multivariate analysis. Dependent variables (maternal hemoglobin and infant birth weight) were examined as continuous variables. Explanatory variables were included as continuous variables (e.g., placental parasitemia, placental monocyte count). A backward stepwise procedure was used, with a removal criterion of $P = 0.2$ and significance set at $P = 0.05$. Non-significant variables were removed sequentially until only significant variables remained.

RESULTS

In the first phase of the study, placental tissue was available for 117 women. Malaria infection was common in this selected group, and 105 placentas had current infection on histology. Placental parasitemia was frequently accompanied by leukocyte infiltrates, which were morphologically and immunohistochemically predominantly of monocyte origin. (Data not shown.) Monocytes ranged from 0–66% (median 3.4%) of all intervillous cells. Intervillositis was defined as a monocyte count ≥ 5%, as this degree of infiltrate was never seen in placentas without malaria infection. Intervillositis was present in 47 placentas (40.2%). Median placental parasite density on histology in placentas with intervillositis was 23%, significantly higher than in placentas without intervillositis, 5.4%, $P = 0.0004$. Monocyte count correlated with placental parasite count ($r = 0.19$, $P = 0.038$), and correlated negatively with birth weight ($r = -0.32$, $P = 0.0004$) and maternal hemoglobin ($r = -0.30$, $P = 0.001$).

On the basis of this first study, we developed a new classification for placental malaria (Table 1) which does not require counting of intervillous cells. Counting of intervillous cells appears to correlate with outcomes of malaria in pregnancy (see below) but is laborious and not suited to widespread clinical application. Instead, placental sections are examined for parasites and for malaria pigment, distinguishing pigment in monocytes and pigment in fibrin deposits as two separate measures. Using these preliminary observations, we performed a larger prospective study, enrolling 357 women who delivered vaginally. Of the women, 165 (46.2%) were primigravidas, 65 (18.2%) in their second pregnancy, and 127 (35.6%) multigravidas. Mean age was 22.4 ± 4.8 years, range 15–45; 29.2% of the women tested were HIV-infected. Malaria infection was detected on peripheral blood film in 55 women (15.4%), on placental film in 72 (20.2%), and on histology in 102 (28.6%). Of the babies, 14.8% were LBW, and 19.9% of the mothers were anemic (hemoglobin < 11.0 g/dL). All 340 women who had information on ANC attendance had attended at least once. Mean number of visits was 5.0 ± 2.2 per woman, and median was 4.5.

In the prospective study, birth weight and maternal hemoglobin, together with prevalence of LBW and of maternal anemia, were compared by placental pathology class, using uninfected placentas as a reference group (Table 1). Class 2 placentas (parasites, monocyte pigment, with or without pigment in fibrin) were associated with lower birth weights and hemoglobin concentrations and higher prevalences of LBW and anemia than all other classes. Babies from placentas with

TABLE 1
Associations between pathology class and birth weight, low birth weight (LBW), maternal hemoglobin, and anemia in the prospective study

Pathology class (number)	Description	Mean ±SD birth weight	Probability compared with class 5 (uninfected)	LBW	Mean ±SD maternal hemoglobin	Probability compared with class 5 (uninfected)	Maternal anemia
1 (13)	Parasites, no pigment in monocytes or fibrin	3,005 ± 294	0.58	0%	12.8 ± 1.7	0.77	7.7%
2 (66)	Parasites, pigment in monocytes ± fibrin	2,700 ± 445	< 0.0001	27.3%	11.8 ± 1.8	0.0001	34.8%
3 (22)	Parasites, pigment in fibrin	2,939 ± 326	0.16	13.6%	12.5 ± 1.8	0.43	18.2%
4 (111)	No parasites, pigment only (past infection)	2,909 ± 416	0.002	14.4%	12.5 ± 1.7	0.095	20.9%
5 (145)	No parasites or pigment (no infection)	3,076 ± 454	REF	11.0%	12.9 ± 1.9	REF	13.5%

Birth weight of babies in class 2 was significantly lower than that of babies in class 3 ($P = 0.023$), class 4 ($P = 0.0018$), and class 1 ($P = 0.026$).
Hemoglobin was significantly lower for class 2 women than for those in class 4 ($P = 0.012$) but not class 1 ($P = 0.09$) or class 3 ($P = 0.10$).

past infection only also had lower birth weights (2,909 ± 416 g) than babies from uninfected placentas (3,076 ± 454 g; $P = 0.002$).

By pairwise correlation, birth weight was significantly negatively correlated with placental monocyte counts (r = –0.14, $P = 0.002$), presence of monocyte pigment (r = –0.27) or fibrin pigment (r = –0.22), and malaria parasitemia on histology (r = –0.19; $P < 0.001$ for all comparisons) but not with peripheral or placental thick film parasitemia. Maternal hemoglobin was significantly negatively correlated with placental monocyte count (r = –0.22, $P < 0.0001$) monocyte pigment (r = –0.15, $P = 0.002$), and fibrin pigment (r = –0.12, $P = 0.013$) but not with histologic parasitemia (r = –0.08, $P = 0.08$) or other measures of malaria infection.

Using our previously derived definition, intervillositis was found in 29 women (8.1%) and was more common in first pregnancy (12.7%) than second pregnancy (4.6%) or multigravid women (3.9%; $P = 0.013$, χ^2). Intervillositis was always associated with class 2 infection in this series. We also recorded the presence or absence of malaria pigment in monocytes. This was found in 69 women (19.3%), of whom 66 had class 2 and three had class 4 (or previous) infection. Monocyte pigment was less frequent in multigravidas (8.7%) than primigravidas (26.6%) or secundigravidas (23.1%; χ^2; $P = 0.001$).

We examined associations between recognized or postulated risk factors and birth weight and maternal hemoglobin concentration (Table 2). By univariate analysis, teenagers; primigravidas; women with few ANC visits; women with anemia, peripheral blood or placental malaria, a history of recent febrile symptoms, intervillositis, or placental monocyte pigment on histology; and women or partners lacking regular income all had infants with significantly lower birth weight. Factors associated with lower maternal hemoglobin included few ANC visits, malaria infection, intervillositis, malaria pigment in monocytes, fibrin pigment, febrile symptoms, and HIV infection (Table 2). Number of doses of antimalarials received from the ANC and maternal bed net use were not associated with birth weight or hemoglobin in this series. Among the women, 18% reported some net use. Febrile symptoms appeared frequently to be attributable to malaria. Of 102 women with malaria, 30 had febrile symptoms, while 24 of 255 without malaria reported fever ($P < 0.001$). Fever was somewhat more common in HIV-infected women (20.6%) than in uninfected women (13.4%; $P = 0.09$, not significant).

The presence of malaria pigment in monocytes was associated with significantly higher rates of LBW (27.1%) and anemia (37.1%) than were found in women in whom it was not detected (11.5% and 16.3% respectively). There also were significantly higher rates of LBW (18.6%) and anemia (25.0%) when placentas had fibrin pigment than when they did not (10.4% and 13.7%, respectively).

Backward stepwise regression was used to look at the independent influence of factors examined in the univariate analyses shown in Table 2 on birth weight and maternal hemoglobin concentration. Gravidity, number of ANC visits, malaria pigment in monocytes, and pigment in fibrin remained significantly associated with birth weight (Table 3). Maternal hemoglobin concentration was associated with ANC visits, HIV infection, placental monocyte count, and recent febrile symptoms after backward stepwise regression. ANC attendance was greater in women receiving the recommended two doses of sulphadoxine-pyrimethamine in pregnancy than in those receiving one dose or none ($P = 0.0002$, *t* test), and in women in first pregnancy than later pregnancies ($P = 0.028$) but was not associated with age, schooling, or subject's or partner's earnings.

DISCUSSION

Understanding how malaria causes LBW and maternal anemia may suggest ways to decrease the impact of malaria in pregnancy.[12] Recent studies have begun both to address the pathologic mechanisms involved[5,9] and to examine correlates between host immune response and pregnancy outcome.[13,14] To explore the associations of parasite counts, host leukocytes, and fibrin pigment with infant birth weight and maternal anemia, we developed a novel staging system for placental malaria (Table 1). Women with placentas containing malaria pigment in monocytes (Class 2) frequently had babies with LBW and were more frequently anemic than other women. We differentiated fibrin pigment alone from monocyte pig-

TABLE 2
Influence of possible risk factors on birth weight and hemoglobin among 357 women

Risk factor	Mean ±SD birthweight (g)		Univariate analysis *P*(*t* test)	Mean ±SD hemoglobin (g/dL)		Univariate analysis *P*(*t* test)
	Present (n)	Absent (n)		Present (n)	Absent (n)	
Age < 20 years	2,858 ± 446 (115)	2,988 ± 442 (232)	0.01	12.5 ± 1.9 (115)	12.5 ± 1.8 (237)	0.77
Primigravid	2,835 ± 461 (164)	3,040 ± 413 (193)	< 0.0001	12.3 ± 1.9 (162)	12.7 ± 1.8 (190)	0.08
Hemoglobin < 11 g/dL	2,811 ± 480 (70)	2,979 ± 436 (282)	0.0049	NA	NA	
4 or fewer ANC visits	2,869 ± 468 (145)	2,987 ± 422 (195)	0.015	12.1 ± 1.7 (143)	12.8 ± 1.9 (192)	0.0009
No antimalarials from ANC	2,879 ± 612 (59)	2,947 ± 401 (287)	0.29	12.5 ± 2.0 (59)	12.5 ± 1.8 (282)	0.99
Placental malaria infection	2,796 ± 423 (102)	3,003 (445) 255	< 0.0001	12.0 ± 1.8 (102)	12.7 ± 1.8 (250)	0.002
Intervillous monocytes ≥ 5%	2,683 ± 412 (29)	2,970 ± 443 (328)	0.0007	10.8 ± 1.4 (29)	12.7 ± 1.8 (323)	< 0.0001
Monocyte pigment present	2,673 ± 461 (69)	3,009 ± 420 (288)	< 0.0001	11.8 ± 1.8 (69)	12.7 ± 1.8 (283)	0.0001
Fibrin pigment present	2,840 ± 421 (193)	3,066 ± 449 (164)	< 0.0001	12.3 ± 1.7 (192)	12.8 ± 1.9 (160)	0.0068
Peripheral malaria infection	2,749 ± 411 (54)	2,981 ± 445 (303)	0.0004	12.0 ± 2.0 (54)	12.6 ± 1.8 (298)	0.02
Fever/chills last week	2,762 ± 460 (54)	2,976 ± 438 (303)	0.0011	11.6 ± 1.8 (53)	12.7 ± 1.8 (299)	0.0001
HIV-1 infected	2,876 ± 405 (93)	2,979 ± 454 (233)	0.058	11.9 ± 1.8 (91)	12.8 ± 1.8 (232)	0.0001
No mosquito net	2,938 ± 445 (303)	2,990 ± 461 (54)	0.43	12.5 ± 1.8 (298)	12.8 ± 1.7 (54)	0.34
Subject not earning	2,906 ± 441 (273)	3,067 ± 452 (83)	0.0041	12.5 ± 1.8 (268)	12.6 ± 1.8 (83)	0.88
Partner not earning	2,709 ± 582 (24)	2,965 ± 439 (302)	0.0075	12.1 ± 1.8 (24)	12.6 ± 1.8 (297)	0.20

* ANC = antenatal clinic

TABLE 3
Factors significantly associated with birth weight and/or hemoglobin after stepwise regression

	Birth weight		Hemoglobin	
	t	*P*	*t*	*P*
Monocyte pigment present	–3.5	0.001	NS	NS
Gravidity	3.2	0.002	NS	NS
Number of antenatal clinic visits	4.6	< 0.001	3.1	0.002
Fibrin pigment present	–2.2	0.030	NS	NS
Percentage placental monocytes	NS	NS	–3.9	< 0.001
Maternal HIV infection	NS	NS	–4.1	< 0.001
Fever or chills in previous week	NS	NS	–3.3	0.001

ment, since they appear to reflect different phases of infection, as shown by significant differences in birth weight and maternal hemoglobin concentration between class 2 and class 3 placentas. Other studies have combined these classes.[8,9] Women with previous infection (class 4) had lower birth weight babies than uninfected women (class 5) did. Fibrin pigment deposition persists for a considerable time after malaria infection. Possible associations between past infection and decreased birth weight are important, as they suggest that "catch up" growth after resolution of infection is incomplete.

Placental monocyte infiltrates appear to be associated with complications of malaria in pregnancy.[4,5,7] Previous studies have used descriptive terminology or semiquantitative measures of infiltrate intensity, or have not distinguished monocytes from lymphocytes.[10] To standardize comparisons, we counted intervillous cell numbers and found that monocyte infiltrates $\geq$ 5% of all intervillous cells were seen only in malaria-infected placentas, and that monocyte numbers were negatively correlated with both birth weight and hemoglobin. Determining monocyte infiltrate density, or point counting,[9,10] allows ready comparability of studies.

We found intervillositis in 12.7% of primigravidas, 4.6% of second pregnancies, and 3.9% of multigravidas, similar to Ordi et al.'s finding of moderate to marked intervillositis in 17% of Tanzanian primigravidas and 2% of other gravidities.[4] The predominance of primiparas suggests that the host-parasite balance is maladapted in first pregnancy but evolves subsequently so that host responses contain the parasite without excessive inflammatory activation.

A number of risk factors for LBW in malaria-endemic areas have been identified or were investigated here, including gravidity, sources of household income, frequency of ANC attendance, recent febrile symptoms, maternal hemoglobin concentration, maternal or placental malaria infection, and antimalarial use (Table 2).[1,11,15,16] In our univariate analyses, these factors were all associated with birth weight. The absence of association between birth weight and HIV infection, use of a bed net, or the prescription of antimalarial drugs through the ANC may reflect the study's relatively small sample size.

Changes observed on histology also were associated with birth weight, including pigment in placental fibrin deposits or in monocytes, and numbers of placental monocytes (Table 2). In The Gambia, birth weights were lower in primigravidas with pigmented placental monocytes, but fibrin pigment was not reported.[6]

Anemia was more common in women with few ANC visits, recent febrile symptoms, malaria infection, and HIV infection but was not associated with bed net (Table 2) or antimalarial use. (Data not shown.) A recent study from Kenya also showed positive associations between malaria, HIV, febrile symptoms, and anemia in late pregnancy.[17] On histology, placental infection, intervillositis, monocyte pigment, and fibrin pigment were all associated with anemia (Table 2).

By multivariate analysis, gravidity, monocyte pigment, fibrin pigment, and number of ANC visits remained associated with infant birth weight (Table 3). The association between birth weight and gravidity is widely recognized,[18] and ANC attendance appears to be associated with increasing birth weight in Africa.[19,20] ANC visits, febrile symptoms, placental monocyte counts, and maternal HIV status remained associated with maternal hemoglobin concentration after multivariate analysis (Table 3). The pathogenesis of anemia in pregnancy in malaria-endemic countries is complex, but malaria, HIV infection, and iron deficiency appear to have key roles.[17,21–23] The association between monocyte count,[7] rather than parasite density, and maternal hemoglobin concentration (Table 3) may be due to differences in the time course of monocyte and parasite numbers. Local placental response to malaria may cause decreased erythropoiesis[24] or may reflect systemic effects of malaria on red cell destruction or survival. Placental tumor necrosis factor (TNF)-α concentrations were increased in Kenyan primigravidas with severe (but not moderate) anemia,[25] and monocyte-derived[14] TNF-α may be having direct or indirect effects on erythropoiesis.

We have categorized placental malaria into groups that are closely associated with important adverse outcomes of malaria in pregnancy. First, we quantified host monocyte infiltrates. These were correlated positively with parasite density and negatively with birth weight, and were inversely associated with maternal hemoglobin in multivariate analysis. Second, placental pathology was divided into classes, depending on the markers of malaria infection observed. Birth weights and hemoglobin levels were lowest when placentas had malaria infection and monocyte pigment detected (class 2). Women with past infection had babies with lower birth weights than uninfected women, whereas babies of women with parasites and fibrin pigment in the placenta (class 3) did not differ significantly from those of uninfected women.

Third, monocyte pigment was detected in 19.6% of placentas, including all 29 women with intervillositis. Women with monocyte pigment had more than twice the rates of LBW and anemia that other women had. Monocyte pigment was associated with dramatic reductions in birth weight (mean 331 g, $P < 0.0001$) and maternal hemoglobin concentration (mean 0.9 g/dL, $P = 0.0001$, Table 2), and remained significantly associated with birth weight in multivariate analyses. Pigment in peripheral blood leukocytes (predominantly in neutrophils) has been associated with poor prognosis of malaria in non-pregnant Thai adults.[26] Placental monocyte pigment may have a similar significance. Understanding the role of monocyte infiltrates in malaria-associated LBW and maternal anemia may lead to improvements in our ability to treat and prevent malaria infection in pregnant women.

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