PLACENTAL CHANGES ASSOCIATED WITH FETAL OUTCOME IN THE PLASMODIUM COATNEYI/RHEUS MONKEY MODEL OF MALARIA IN PREGNANCY

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Abstract. Term placentas collected surgically from seven Plasmodium coatneyi–infected rhesus monkeys, one abortion, and five controls were evaluated histopathologically. The placentas from Plasmodium-infected dams had more significant pathologic changes than those from controls for six parameters (P < 0.05) and higher numbers of activated (LN5 + Zymed) macrophages in the intervillous space (IVS) (P = 0.0173). Total parasite load (TPL) was defined as the sum of all weekly peripheral infected red blood cell counts for each trimester and for the entire pregnancy. High first trimester PLs were more likely to result in fetal demise (P = 0.0476) or increased placental damage in surviving infants. As trimester 2–3 TPL increased, so did the number of activated macrophages (P < 0.05) and the total malaria pigment scores (P < 0.05). Low birth weight (LBW) and intrauterine growth retardation (IUGR) were associated with high pigment scores and high numbers of activated macrophages in the IVS. High placental damage scores were not associated with IUGR, LBW, or early infant mortality.

INTRODUCTION

Infection of pregnant women with Plasmodium falciparum has been associated with abortion, intrauterine growth retardation (IUGR), low birth weight (LBW), neonatal mortality, and congenital infection. We have recently established that the pregnant rhesus monkey inoculated with P. coatneyi is an excellent animal model of this disease process, demonstrating these same adverse fetal/infant outcomes.

Examination of the placenta is essential to explaining abnormal fetal and neonatal outcome. However, only a few reports have included a histopathologic examination of the placentas from Plasmodium-infected women. Studies that have reported placental histopathologic findings were primarily descriptive and did not relate their findings to maternal morbidity and fetal/infant outcome. Malaria induced placental changes identified in these studies included focal syncytiothrophoblast necrosis, irregular thickening of the trophoblast basement membrane, the presence of parasitized erythrocytes and aggregates of leukocytes (including macrophages) within the intervillous space, macrophages containing malaria pigment, malaria pigment deposited in fibrin and fibrinoid, fibrinoid deposits, and fibrinoid necrosis of villi.

Most animal studies of malaria during pregnancy have been conducted in rodents with P. berghei. Unfortunately, the reproductive systems of rodents do not represent the human reproductive system very well. In contrast, the nonhuman primate, unlike rodents, has a villous hemochorial placenta and fetal organogenesis comparable to the human. Rodents have also not proven to be useful models of congenital malaria.

To fully establish the P. coatneyi-infected pregnant rhesus monkey as a comprehensive model of malaria during pregnancy, the results of the pathologic evaluation of the placental tissues surgically collected from seven term pregnancies and one first trimester intrauterine death are reported here. In addition, these results are correlated with maternal parasitemia, morbidity, and fetal/infant outcome.

MATERIALS AND METHODS

General study design. Animals. The clinical details of this study have been published. Briefly, 10 pregnant, malaria-naive, Indian-origin rhesus monkeys (Macaca mulatta) were inoculated intravenously with P. coatneyi during the first trimester between gestational days (GDs) 30 and 54. These 10 females had been born at the Tulane Regional Primate Research Center (TRPRC) and therefore had no previous exposure to malaria parasites. Three dams were primigravidas (M181, L226, and L422), one was a secundigravida (L414), and six were multigravidas (D673, E412, J653, L412, L434, and M488). In a subsequent study, another malaria-naive primigravid monkey (N833) was inoculated with P. coatneyi on GD 61. Although the results of this animal’s inoculation were not previously published, the placenta from this animal was included in this report.

Rhesus monkey gestation averages 165 days (± 10 days). Thus, the first trimester in the rhesus monkey is from GD 0 to 55, equivalent to GD 0–90 in the human; the second trimester is from GD 56 to 110, equivalent to GD 91–180 in the human; and the third trimester is from GD 111 to 165, equivalent to GD 181–270 in the human.

Inoculation and delivery scheme. Initially, fresh blood from a P. coatneyi-infected rhesus monkey with a parasitemia of 7% (10^6 infected red blood cells [IRBCs]) was inoculated into four monkeys (M181, M488, L412, and E412) during their first trimester (< GD 55). Because three of four inoculations resulted in two abortions and surgical clearance of one dead fetus at 7–10 days post-infection (PI) (M181, M488, and L412), the size of the effective inoculum was reduced. Frozen blood from the same donor (10^8 IRBCs) was used to inoculate six additional monkeys (D673, J653, L226, L414, L422, and L434). All six became parasitemic within 14 days PI, and along with monkey E412, carried their infants to term. Monkey N833 was inoculated with a similar frozen inoculum (10^8 IRBCs) from a different donor and also delivered a viable-term infant.

Seven infants were delivered by elective cesarean section before the onset of labor (GD 155) to ensure collection of the placenta, which is otherwise consumed by the dam. Delivery prior to labor also ensured that the placentas did not contain lesions associated with labor and delivery.

Material and her infant’s placenta was therefore eliminated from the
Schematic of a Full Thickness Cotyledon

Figure 1. Schematic of a full-thickness section through a placental cotyledon. A main stem villus containing fetal vessels extends from the chorionic plate (fetal side) to anchor on the maternal side at the basal plate. The villous "tree" extends into ramifications of intermediate and then terminal villi, which are seen as cross sections in the figures that follow. These villi contain fetal vessels and are surrounded by the intervillus space, which contains maternal blood. The syncytiotrophoblast layer, which surrounds each villus, is the definitive barrier between the fetal and maternal blood supply.

study. Infants were weighed, measured, and returned to their dams within 24 hr of delivery. Infants rejected by their mothers were nursery-reared. Surgery was performed on monkey L412 to collect the fetoplacental tissues when fetal demise was diagnosed in utero at 37 days gestation. Five monkeys treated in the same manner, with the exception of Plasmodium inoculation, served as controls.

Pathologic examination of placentas. Tissue collection. Placental tissues were collected at the time of elective cesarean section, weighed, and examined grossly. A cross-sec-

Table 1

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<th>3</th>
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<td>2 per 100</td>
<td>3 per 100</td>
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<td>60 affected villi</td>
<td>90 affected villi</td>
<td>≥ 90 affected villi</td>
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<td></td>
<td>Basal</td>
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<td>&gt; 20%, ≤ 40%</td>
<td>&gt; 40%, ≤ 60%</td>
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<td>&gt; 20%, ≤ 30%</td>
<td>&gt; 30%</td>
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<tr>
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<td>≤ 15%, ≤ 30%</td>
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<td>Malaria pigment (hemozoin)#</td>
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<td>rarely seen or small amounts in 1–2 fields</td>
<td>≤ 15%, ≤ 30% multiple fields with specific focal areas</td>
<td>&gt; 50% abundant pigment throughout area</td>
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<td>Pigment in macrophages**</td>
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<td>5–10</td>
<td>11–30</td>
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<tr>
<td></td>
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<td>4–8</td>
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<td>Intervillous space (IVS)</td>
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<td>≤ 1/field</td>
<td>≤ 1.5–2/field</td>
<td>≤ 3/field</td>
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<tr>
<td>Parasites in sections††</td>
<td>no IRBC’s</td>
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<td>1 IRBC/1–2 HPF</td>
<td>≥ 2 IRBC’s/HPF</td>
<td>numerous IRBC’s</td>
</tr>
</tbody>
</table>

* Number of affected villi per 100 villi examined in randomly chosen microscopic fields.
† Number of fetal villi affected in one area.
‡ Greatest diameter.
§ SD = percentage of villi affected per 40X field. Chorionic plate SD = percentage of plate affected when entire syncytiotrophoblast layer examined.
# Amount of pigment found in examined fields.
** Highest number of pigmented macrophages found in any 40X single field or the mean number of pigmented macrophages per field.
†† Number of infected RBC’s (IRBC) per high power field (HPF).
tion was removed during surgery and blotted onto five slides to make thick and thin placental blood smears. Immediately following surgery, sections 3–4 mm in width were taken randomly from five different cotyledons by starting at the maternal decidua and cutting through the full thickness of the cotyledon, including the fetal chorionic plate (Figure 1), and placed into tissue cassettes. In addition, five sections

Figure 2. (a) Matrix-type fibrinoid (F-arrows) replacing villous stroma termed, “fibrinoid necrosis of villi” (H&E, 100X). (b) Perivillous fibrinoid (arrow) replacing the syncytiotrophoblast layer which surrounds a villus.

Figure 3. (a) A “distinct fibrinoid lesion” (DFL) with fibrinoid necrosis of >120 villi (grade 4). Fibrinoid is deposited within and between villi, obscuring the villous outlines. There are inflammatory cells, macrophages, malaria pigment, and increased numbers of cytotrophoblast cells. A clear demarcation exists between affected villi (left), and unaffected villi (V) (right) (H&E, 10X). (b) Higher magnification (40X) of the DFL in Figure 3a. with fibrinoid (F), deposits of malaria pigment (arrowheads), and inflammatory cells (arrows).
of the umbilical cord and one strip of choioamniont membrane, rolled with cut end down, were placed into cassettes. Tissues were fixed in 10% neutral-buffered formalin for 24 hr for histopathologic analysis or in Streck Tissue Fixative (STF) (Streck Laboratories Inc., Omaha, NE) for immunohistochemical analysis. Tissue blocks were sectioned at 5 μm and stained with hematoxylin and eosin, periodic acid-Schiff, and Giemsa.

Scoring of lesions. The scoring was completed by one pathologist without knowing the identity of the animals. All sections were histologically evaluated using a list of parameters graded from 0 to 4, with zero representing “none”, one being minimal, and 4 the most severe. For each case, the parameter score was the sum of the scores given for each section. For example, if the score for chorionic plate thrombosis was 4 for each of five cotyledon sections examined, the severity score would be 20 for this parameter. Only distinct fibrinoid necrosis lesions and infarcts were scored as a sum of the scores for all individual lesions rather than sections. The criteria are described below and summarized in Table 1.

Description of parameters evaluated. Fibrinoid deposits (FD). Fibrinoid is an acellular, intensely eosinophilic material of which there are two types located in placental tissue. These are 1) fibrin-type fibrinoid, a blood clotting product that is free of extravillous trophoblast cells and usually in contact with the intervillous space (IVS); and 2) matrix-type fibrinoid, which are embedded varying numbers of extravillous trophoblast cells and which itself is a secretory product of these cells. When matrix-type fibrinoid replaces villous stroma, leaving an intact trophoblastic surface, it is referred to as fibrinoid necrosis of villi (Figure 2a). Perivillous fibrinoid is a matrix-type fibrinoid which replaces the trophoblastic cover of the villus (Figure 2b).

Distinct fibrinoid lesion (DFL). This is a term coined by the principle investigator. This parameter is qualitatively and quantitatively different from the lesion fibrinoid necrosis of villi as described by other investigators (see fibrinoid deposits). The DFL is an area of fibrinoid necrosis involving > 30 fetal villi viewed in cross section. The affected area has a complete loss of normal villous architecture. Fibrin-type fibrinoid is deposited within villi and matrix-type fibrinoid is deposited between villi, obscuring the villous outlines (Figures 3a and b). Adjacent tissues such as the basal plate, marginal sinus, and the chorionic plate are often, but not always, involved. The DFLs may include infiltrates of inflammatory cells and macrophages, deposits of malaria pigment, and often contain increased cytotrophoblast cells.

Infarcts. Marginal infarcts were defined as areas of ischemic necrosis of villi adjacent to the marginal sinus of a cotyledon, while basal infarcts originated adjacent to the basal plate and extended toward the amnion. Infarcts were graded according to location and size, measured across their greatest diameter using a reticle (Table 1).

Chorionic plate thrombosis (CPT). This refers to thrombi within fetal vessels of the chorionic plate often leading to thromboses (Figure 4a 1 and 2, 4b 1 and 2), which extend into the major ramifications of fetal vessels within stem villi. The lesion resulted in an infarction like lesion in the chorionic plate itself, but was not considered to be a true infarction, which only occurs when the maternal blood supply is disrupted.

Syncytiotrophoblast disruption (SD). The syncytiotrophoblasts form a continuous cell layer which covers the villi and chorionic plate. The syncytiotrophoblast layer was examined for areas of disruption (Figure 5).

Inflammation. The severity of polymorphonuclear and mononuclear infiltrates was evaluated within the IVS, villi, umbilical cord, choioamniotic membrane, DFL, and surrounding vessels in the decidua (perivascular cuffing). The pathologist used previous control studies to establish guidelines for levels of inflammatory cells considered as acceptable numbers seen in preterm delivered placentas.

Placental damage score. This is the sum of all scores for DFL, infarcts, CPT, fibrinoid deposits, syncytiotrophoblast disruption, and inflammation in all sections examined for a case.

Malaria pigment (hemozoin). This is an extracellular, brown to black, iron-negative, birefringent byproduct of the metabolism of hemoglobin by Plasmodium. It was evaluated within the decidua, basal plate, villi (Figure 5), intervillous space, maternal and fetal RBCs, trophoblasts, fibrinoid deposits, and chorionic plate.

Macrophages containing malaria pigment. These (Figure 6) were evaluated in the decidua, basal plate, IVS, villi, DFL, and fetal vessels. The entire decidua or basal plate was scanned at 40× and the highest number of pigmented macrophages visualized in a single field was used to determine the score (Table 1).

Total pigment score. This is the sum of the scores for pigment within fibrin and pigment within macrophages in all sections examined for a case.

Placental parasitemia. A cross-section of placental cotyledon was removed during surgery and blotted onto five slides to make thick and thin placental blood smears that were stained with Giemsa. The IBRC were counted in 10 microscopic fields each containing approximately 200 RBCs (1,000× magnification) and recorded as a percent of RBCs parasitized.

Parasites in sections. The IRBCs were identified by light microscopy in Giemsa-stained placental sections by focusing up and down on RBCs within the IVS, fetal vessels (Figure 7), and umbilical cord at 100× under oil immersion optics.

Peripheral parasitemia/parasite load. To better facilitate an evaluation of the impact of circulating parasites on placental lesions and fetal outcome, coupled with the gestational time when they occurred, the percent of maternal IRBC was determined weekly. The percent of maternal IRBCs was determined by counting 10 microscopic fields (1,000× magnification) each of which contained approximately 200 RBCs. The RBC count on the same day was used to calculate the number of IRBCs/mm3 of blood. No attempt was made to count individual parasites within RBC. The total parasite load (TPL) was determined to be the sum of all weekly IRBC counts during pregnancy. This sum was also calculated for each trimester of pregnancy with T1, T2, and T3 representing the first, second, and third trimesters, respectively. These figures appear in Table 2.

Activated macrophages (AM). These were identified by immunohistochemical staining with monoclonal antibodies to LN-5 (Zymed) (Figure 8). For each case, the macrophages...
Figure 4a. (1) A full thickness section of a cotyledon from a *Plasmodium*-infected monkey (E412). Chorionic plate thrombosis is severe in 2 areas (arrows). Computerized image (H&E, 2X). (2) A higher magnification of the area under the arrow located outside the section in Figure 4b.1. Chorionic plate thrombosis (CPT) resulted in ischemia and necrosis which developed into an infarction-like lesion. Three fetal vessels (FV) are shown on the surface of the chorionic plate and the fetal villi (V) are seen in cross section below. Computerized image (H&E, 10X).
PLACENTAL MALARIA IN MONKEYS

FIGURE 4b. (1) A full thickness section of a cotyledon from a control monkey illustrating a normal chorionic plate (arrowheads) with a fetal vessel (arrow) on the surface. Cross sections of fetal villi lie beneath the chorionic plate. Compare with Figure 4a.1 (H&E, 2X). (2) A higher magnification of the area of normal chorionic plate indicated by the large arrow in Figure 4b.1. There is a large fetal vessel (FV) above the chorionic plate (CP). Normal cross sections of fetal villi lie beneath the plate. Compare with Figure 4a.2 (10X H&E).

(stained red with amino ethyl carbazole (AEC) were counted in 10 randomly selected high-power fields (HPF) (100X oil immersion) within the IVS and this total was divided by 10 to establish the mean. The actual mean was used as the score for each section. Only HPFs containing substantial amounts of blood within the IVS were evaluated.

Fetal macrophages (Hofbauer cells). These were determined by the same method by counting immunostained macrophages in ten randomly selected terminal villi. Each villus examined was roughly 0.06 × 0.06 mm in diameter.

Immunohistochemistry. Placental tissues were fixed in Streck Tissue Fixative (STF; Streck Laboratories, Inc. Omaha, NE) and activated macrophages were identified using LN-5 (Zymed Laboratories, South San Francisco, CA) using routine immunohistochemistry methods.

Statistical analysis. The resulting median scores from the
FIGURE 5. A fetal villus containing fibrinoid (F) and deposits of malaria pigment (arrowheads). A macrophage (small arrow) containing malaria pigment lies adjacent to an area of syncytiotrophoblast disruption (large arrow). The syncytiotrophoblast cell layer is indicated (ST) (100X H&E).

FIGURE 6. Macrophages containing malaria pigment (arrows) in the intervillous space. Villi (V) and the intervillous space (IVS) are indicated (H&E, 100X).
evaluation of the parameters for *Plasmodium*-infected placentas (Med$_a$) were compared to control placentas (Med$_b$) using the Mann-Whitney test. Means and standard deviations (SD) were calculated for the number of activated maternal macrophages and Hofbaur cells and placental weights. Differences between means were tested using the student t-test for independent samples. When presented in the results, the SD follow the mean with a ± sign. Correlations between maternal clinical parameters, placental parameter scores and infant outcomes were compared using the Spearman rank correlation. Comparisons made between selected parameters and combinations of the following variables were made using the Fisher Exact test: IUGR (presence or absence), fetal demise (alive or dead), early infant mortality (yes or no), parasite-infected fetal RBC (presence or absence). All tests were two-tailed with the level of significance set at $P \leq 0.05$.

**RESULTS**

**Placental weight.** Placental weights in the *P. coatneyi*-infected group were lower than in non-infected controls ($t = 2.5548$, degrees of freedom [df] = 15, $P = 0.0220$). The placental weight range was 83–115 grams with a mean ± SD of 104.28 ± 12.80 grams. The average placental weight decreased ($r = 0.8524$, $P < 0.05$). As parasite load during T-2 and T-3 increased, so did the number of activated macrophages in the intervillous space ($r = 0.7500$, $P < 0.05$). As total pigment increased, infant weight decreased ($r = 0.69$, $P < 0.05$). The number of activated macrophages was lower in the infected group than in non-infected controls ($t = 3.22$, $P < 0.05$).

**Table 2**

Parasite load during each trimester coupled with maternal red blood cell count/anemia, fetal outcome and placental lesion scores

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<th>p3</th>
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<th>wt g/l</th>
<th>LRBC</th>
<th>A</th>
<th>IUGR</th>
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*p1–p3 = parasite loads during each trimester × 10,000; pwt = placental weight in grams (control mean = 123.9); TP = total parasite load during pregnancy × 10,000; bwt = birth weight in grams × 100 (control mean = 4.56); LRBC = lowest red blood cell count × 101,000; status = status of infant; abort = fetal demise; dead = early infant mortality (3–33 days); and alive = still alive at this time; C = congenitally infected with malaria; A = anemia with r = recovery of normal RBC count (>4.5) and s = no recovery (<4.5) during gestation; wt g/l = weight gain or loss during pregnancy (control mean = +17.0625); IUGR = intrauterine growth retardation; m = mild during late gestation; s = severe; PD = placental damage score; PIG = total pigment score; INF = inflammation score; shaded background = highest scores; MAC = average number of activated macrophages/HPF. Scores reflect total for all sections examined.

There was a significant association between parasitemia (high or low) occurring during T-1 and high placental damage scores ($r = 0.7275$, $P < 0.05$). As total pigment increased, infant weight decreased ($r = 0.8524$, $P < 0.05$). As macrophages increased, infant weight decreased ($r = 0.8524$, $P < 0.05$). As parasite load during T-2 and T-3 increased, so did the number of activated macrophages in the intervillous space ($r = 0.7500$, $P < 0.05$).
Figure 8. Activated macrophages (LN5+) appear as dark black cells (arrowheads) within the intervillous space (IVS) adjacent to fetal villi (V) in the placenta from a Plasmodium-infected monkey (AEC with H&E counterstain, 100X).

in controls was 123.9 ± 20.90 grams. The placenta weighing 83 grams belonged to the infant with the most severe growth retardation and a birth weight of 334 grams (control mean = 466 grams). This placenta and one other were mildly circumvallate (Figure 9); however, the significance of this finding is unknown. All other gross parameters in the seven term placentas appeared normal.

Placental parasitemia and parasites in sections compared to peripheral parasitemia. In all seven cases, the percentage of IRBC in the placental blood smears was the same as that of the dam’s peripheral blood on the day of delivery. Although IRBCs could be identified within the IVS of placental tissue sections in all seven cases, there was no increased accumulation of IRBC within the IVS nor were there notable IRBC adhering to the surface of the syncytiotrophoblast layer of the villi.

Histopathology of cesarean-delivered term placentas from infected versus non-infected dams. The placental histopathology from P. coatneyi-infected monkeys (Figures 2–8) resembled that seen in P. falciparum-infected humans. The seven placentas from P. coatneyi-infected dams had more significant pathologic changes than did the placentas from 5 control animals when the median scores were compared for each parameter using the Mann-Whitney test (Figure 10 a–f). The distinct fibrinoid lesions (Figure 3a and b) were more numerous and severe when the median scores for malaria-infected (MedM = 31) were compared with that of the controls (MedC = 9) (P < 0.05). Fibrinoid deposits (Figure 2a and b) were more severe than those seen in controls (MedM = 54 versus MedC = 26) (P < 0.01) (Figure 10c). One of the most significant findings was the increased infiltrates of inflammatory cells (Figure 3b) in P. coatneyi-infected placentas (MedM = 31) compared with controls (MedC = 9) (P < 0.01) (Figure 10d). Malaria placentas had significantly increased scores for disruption of the syncytiotrophoblast layers of both villi (Figure 5) and the chorionic plate (MedM = 18 versus MedC = 11) (P < 0.01). The placental damage scores for malaria cases (MedM = 154) were significantly higher than that of the controls (MedC = 47) (P < 0.01) (Figure 10e). The mean number of activated macrophages (LN-5+) in the IVS of malaria placentas (6.3 ± 3.359) (Figure 8) was significantly higher than the mean for controls (2.2 ± 1.272) (t = 2.849, df = 10, P = 0.0173) (Figure 10f). Activated Hofbaur cells (fetal macrophages) were significantly increased in malaria placentas (mean = 3.7 ± 1.521) over controls (mean = 1.7 ± 0.303) (t = 4.353, df = 10, P = 0.0014).

Histopathology from Plasmodium-induced intrauterine death. Upon ultrasonographic determination of fetal death at seven days PI, 37 days gestation, the placenta and fetus were surgically recovered from monkey L412. This monkey had quickly developed active malaria with a parasitemia of 21,915 parasites/mm³ of RBCs. The placenta was evaluated, but did not receive parameter scores. The etiology of fetal death appeared to be related to severe acute placental infarction. Within the attached uterine tissue (endometrium) there was infarction with inflammatory cell infiltrates and sequestration of parasitized erythrocytes in endometrial cap-
illaries. The number of capillaries with sequestration increased towards the placental tissue. There was infarction of the basal plate with mild neutrophilic infiltrates. The intervillous space contained deposits of fibrinoid material. These deposits contained parasitized erythrocytes and malaria pigment. Macrophages associated with these areas contained phagocytized *P. coatneyi* and malaria pigment. These areas were associated with fibrinoid necrosis of villi.

**Statistical analysis of parameters studied versus pregnancy outcome in *Plasmodium*-infected dams.** Associations between placental parameter scores, the sums of parameter scores, total parasite load during pregnancy, parasite load during individual trimesters, and various fetal outcomes were compared by the Spearman rank correlation (rs). Only significant results or those showing a high correlation are reported (Table 2).

Monkeys M488, L412, E412, and J653 were inoculated during the first trimester (T-1) and all four experienced parasitemic episodes during T-1. Three had high T-1 parasite loads of greater than 100,000 IRBCs/ml of blood (M488, L412, and E412), while monkey J653 had low levels (12,000). Two of the three mothers with high T-1 parasite loads (M488 and L412) aborted. The placental lesions from monkey L412 are described above. The placentas from the viable births of monkeys E412 and J653 were the only two of seven term placentas with significant CPT (Figure 4a). Parasites in T-1 were significantly associated with CPT (r = 0.8321, P < 0.05) (Figure 11). These two placentas also had the highest overall placental damage scores. There was a significant association between parasitemia (high or low) occurring during T-1 and high damage scores (r = 0.7572, P < 0.05). Chorionic plate thrombosis scores were positively associated with DFL scores (Figure 3a and b) (r = 0.6875), but this did not reach significance. The third abortion in this study (M181) was inoculated late in T-1 (GD 51) resulting in a high parasite load (65,000) early in the 2nd trimester (GD 58–60). Parasite inoculations during T-1, resulting in high parasite load during early gestation, were more likely to result in fetal demise (P = 0.0476).

Low birth weight and IUGR were associated with high pigment scores and high numbers of activated macrophages in the IVS. As the sum of the parasite load during T-2 and T-3 increased, so did the number of activated maternal macrophages (r = 0.7500, P < 0.05) (Figure 8) and the total pigment scores (r = 0.7500, P < 0.05) (Figure 12). The relationships between total pigment scores, activated maternal macrophages, and infant weight were highly significant. As total pigment increased, infant weight decreased (r = -0.8524, P < 0.05). This relationship became even stronger when pigment in fibrin (r = -0.9266, P < 0.05) (Figure 5) and pigment in macrophages (r = -0.9266, P < 0.05) (Figure 6) were examined independently. As macrophages increased, infant weight decreased (r = -0.8524, P < 0.05).

Monkeys N833, L414, and L226 had the highest levels of activated macrophages and the highest total pigment scores. Monkeys N833 and L226 gave birth to infants that died at three and five days of age. The infant born to L226 had severe IUGR, LBW, and the lowest placental weight. Monkey L414 delivered a LBW infant with IUGR that survived, and congenital malaria was diagnosed at three months of age. This infant’s placenta contained the highest level of activated macrophages in our study (Table 2).

As total parasite load increased, the scores for total in-
FIGURE 10. (a-f) A comparison of the distribution of parameter scores between the five control and eight Plasmodium-infected placentae. Each severity score represents the sum of the total points assigned for each slide examined for a given parameter. Five full-thickness sections of different cotyledons were examined for each case. For each parameter examined, the mean or median scores were significantly higher ($P < 0.05$) for placentae from Plasmodium-infected dams than those from the control monkeys.
Figure 11. First trimester parasitemia, regardless of level, was significantly associated with high scores for chorionic plate thrombosis (CPT). See Figure 4a ($P < 0.05$) and total placental damage ($P < 0.05$). This finding is illustrated by the results observed with monkeys E412 and J653. Monkey E412 had a very high parasite load during T-1 and monkey J653 had a low parasite load during T-1. Other T-1 parasitemias resulted in abortion ($P = 0.0476$) (see Table 1).

Figure 12. High parasite loads during the latter two-thirds of pregnancy were significantly associated with high total-pigment scores ($P < 0.05$) and increased numbers of activated macrophages in the intervillous space ($P < 0.05$) (see Figure 8). As pigment increased, infant birth weight decreased ($P < 0.05$) and as the number of activated macrophages increased, infant birth weight decreased ($P < 0.05$). Very low-birth-weight infants were born to L226 and L414 (see Table 1).
scores were not associated with IUGR ($P = 0.4286$), LBW ($r_0 = -0.2594$), or early infant mortality ($P = 1.000$).

As total parasite load increased, the scores for IRBCs observed within the IVS also increased ($r_i = 0.8214$, $P < 0.05$). The number of fetal IRBCs in vessels of placental villi showed a positive correlation with total parasite load, but this did not reach significance ($r_i = 0.6736$). Fetal IRBCs were identified in vessels of placental villi (Figure 7) in the four monkeys with the highest total parasite loads (> 150,000 parasites/mm$^3$ of RBCs), but not in the three with low total parasite load (< 55,000 total). This was a significant finding ($P = 0.0286$). Interestingly, as activated maternal macrophages increased within the IVS, the number of fetal IRBCs identified in vessels of placental villi increased ($r_i = 0.7769$, $P < 0.05$).

The two monkeys with the highest parasite loads during the third trimester, N833 and L226, had the lowest placental weights (88.97 and 82.68 grams, respectively). Although there was a positive association, ($r_i = -0.6786$), it did not reach significance. These infants died at three and five days of age.

**DISCUSSION**

We used the *Plasmodium coatneyi*-infected pregnant rhesus monkey model to evaluate the role of the placenta in the pathogenesis of the adverse effects of malaria on pregnancy outcome. We followed maternal parasite load, maternal RBC counts, and fetal development throughout the course of infection. When combined with the results of thorough placental histopathology (from cesarean section–delivered placentas that were free from the effects of parturition) and pregnancy outcome, important insights were derived.

Malaria during pregnancy in non-immune women is highly associated with abortion and stillbirth. For instance, in one study 13 of 14 women presenting with malaria in T-1 aborted, as opposed to eight of 19 presenting in T-2. In our model, all three abortions occurred in dams with high parasitemia in T-1, as did the two most severely damaged placentas. Thus, the degree and timing of parasitemia may be important factors in inducing abortion. Placental infarction appears to be one pathologic mechanism that leads to abortion. Infarction may be secondary to obstruction of endothelial vessels with parasitized RBCs. While worse pregnancy outcomes have been reported in primigravidas, abortion as a result of infectious disease during early gestation is not unique to malaria. Abortion in dams with documented peripheral blood parasitemia in T-1 appears to outweigh the effect of parity on pregnancy outcome, important insights were derived.

Malaria during pregnancy in non-immune women is highly associated with abortion and stillbirth. For instance, in one study 13 of 14 women presenting with malaria in T-1 aborted, as opposed to eight of 19 presenting in T-2. In our model, all three abortions occurred in dams with high parasitemia in T-1, as did the two most severely damaged placentas. Thus, the degree and timing of parasitemia may be important factors in inducing abortion. Placental infarction appears to be one pathologic mechanism that leads to abortion. Infarction may be secondary to obstruction of endothelial vessels with parasitized RBCs. While worse pregnancy outcomes have been reported in primigravidas, abortion as a result of infectious disease during early gestation is not unique to malaria.

While high parasitemia in T-1 is associated with abortion, high parasitemia in T-2/3 is associated with IUGR, LBW, and congenital infection. In our study, these outcomes correlated with high levels of activated macrophages within the intervillus space and heavy deposits of malaria pigment.

The mechanism by which placental malaria contributes to IUGR and LBW is not fully understood. In response to *Plasmodium*, activated placental macrophages could induce placental damage, both mechanical and functional, through the release of cytokines such as interferon-γ and tumor necrosis factor-α. A strong local (placental) Th1 response to *Plasmodium* such as that observed in a primary infection of a naive monkey may compromise pregnancy in a manner such as we and others have postulated. In our study, areas of syncytiotrophoblast disruption were often associated with increased aggregates of macrophages (Figure 5) and inflammatory cells, as has been observed in human studies. While increased malaria pigment may have a primary role in contributing to poor fetal outcome, it is more likely that it serves as a marker for high parasitemia late in gestation.

Because it is not feasible to examine blood smears daily, levels of placental hemazoin may provide the investigator with a marker of severe parasitemia sometime during the later half of pregnancy. This can easily be missed clinically. High levels of pigment in the placenta should alert the physician that the infant may be at high risk for malaria induced complications. However, low pigment scores will not allow the clinician to rule out severe parasitemia during early pregnancy. High pigment scores would not be expected to be associated with parasitemia during the first trimester because fibrin, where most malaria pigment is deposited, is not a major component of a first trimester placenta. As the placenta matures, fibrin becomes a more prominent feature. Another factor affecting hemazoin levels might be the length of time it remains in placental tissue. Although it is thought to remain for many months, it may not last the entire gestation. Some women with documented peripheral blood parasitemia during the first half of gestation had no placental malaria pigment at term delivery. This is illustrated by monkey E412 who had one of the highest total parasite loads in this study, but a low total pigment score. Seventy-five percent of the parasites in this monkey occurred during T-1. Although this placenta had high placental damage scores, the infant had no complications. In order to use hemazoin as a diagnostic tool, several placental sections must be examined. Another method, which we have not yet explored in the monkey model, would be to quantitate the hemazoin levels in placental tissue by spectrophotometric or fluorometric assays.

Intuitively, one would expect high placental damage scores to be associated with the worst infant outcome. This was not the case in this study. The two placentas with the highest placental damage scores (J653 and E412) were not associated with IUGR, LBW, or congenital infection. One common factor, unlike the other six term pregnancies, was that both monkeys had parasitemias during the first trimester. Monkey E412 had a high parasite load during the first trimester and monkey J653 had a low parasite load during T-1. Thus, it appears that any parasites occurring during T-1 can result in extreme placental damage. In monkeys E412 and J653, the resulting damage was manifested as thrombosis of fetal vessels in the chorionic plate and stem villi with extensive DFLs that appeared to result from the vascular occlusion. Thrombosis of chorionic vessels is known to occur in placental toxoplasmosis and in infections with cytomegalovirus as well.

Fibrinoid necrosis of villi was a prominent finding in *Plasmodium*-infected placentas; however, it was not a hallmark of poor infant outcome. Fibrinoid necrosis and distinct fibrinoid lesions of the placenta were defined in a manner which is qualitatively different than that reported in the human placental malaria literature. Fibrinoid necrosis is known to be a normal component of term placental villi; however, extensive fibrinoid necrosis is abnormal. In an effort to differentiate normal fibrinoid from a “distinct fibrinoid lesion”, we only considered large areas of necrosis
Sequestration of Plasmodium-infected erythrocytes was not observed in the placentas in this study. Neither cytoadherence of IRBCs to syncytiotrophoblasts nor increased concentration of IRBCs in the IVS was seen. If sequestration does occur in the monkey model, our failure to observe it may be the result of small sample size. Sequestration may also be associated with labor and vaginal delivery while the placentas in this study were cesarean derived. Sequestration may be a terminal event. As uterine contractions begin, disruption of blood flow to the placenta could lead to a more stagnant flow in circulation with IRBC accumulation in the IVS. Very high levels of IRBC occur in only about 25% of placentas from malaria-infected women, especially primigravidae. After a critical review of the literature, it is our opinion that the term “sequestration” has been used differently in reference to the placental intervillous space than is commonly used in reference to the microvasculature of the brain. Originally, “placental sequestration” referred to the observation that higher levels of parasitemia occurred within blood collected from the intervillous space or in IVS blood in histopathological sections than in the peripheral blood of the mother at the time of delivery. Clear evidence has been presented by many investigators demonstrating an increased accumulation of IRBCs in the IVS in vivo. The general term sequestration is often used by pathologists in this manner. On the other hand, malariologists have a more specific definition for sequestration, which describes a receptorligand driven phenomenon whereby IRBCs adhere to the vascular endothelium. An example is cerebral malaria. The distinction between these two phenomena has now been lost in that placental sequestration has been interpreted by some investigators to describe an event whereby IRBCs preferentially adhere to the syncytiotrophoblast cell layer. Binding of IRBCs to syncytiotrophoblast has rarely been observed in vivo. This type of placental sequestration phenomenon was first explored by Bray and Sinden in 1979. Based on the literature concerning sequestration as an endothelial cell event, Bray and Sinden explored the possibility that this same type of phenomenon occurred in the placenta by histopathology and electron microscopy. Their studies did not reveal any significant number of IRBCs lining the syncytiotrophoblast. This type of in vivo sequestration has not been described by others. However, there are many reports that describe specific receptor driven placental sequestration in vitro, and the mechanism by which IRBC accumulate within the IVS is now being advanced as a receptor driven event.

In an ex vivo model, Fried and Duffy used human placental sections and demonstrated that IRBCs from pregnant women bound exclusively to chondroitin sulfate A (CSA), a receptor that occurs on the surface membrane of placental syncytiotrophoblasts, while no IRBCs from nonpregnant women were bound. Maternal IRBCs from the peripheral circulation had a CD-36 binding phenotype, a CSA-binding phenotype, or a mixed phenotype. There was no adhesion of placental IRBC to CD36, thrombospondin (TSP), or intercellular adhesion molecule-1 (ICAM-1) in vitro. The role of variant antigens on the surface of infected erythrocytes in cytoadhesion to purified CSA, CD36, and ICAM-1 was explored using IRBCs from placentas, maternal peripheral blood, and blood from malaria-infected children. In contrast to Fried and Duffy, Beeson and others found that the phenotype of IRBC that bind to CSA may not be exclusively localized to the placenta. The results indicated that 80% of midpregnancy peripheral blood isolates adhere to CSA as did 78% of placental isolates and 13.6% of child isolates. Four placental isolates did not bind to CSA. Adhesion to CD36 occurred in 65% of child isolates, 47.1% of peripheral maternal isolates, and 17.6% of placental isolates. There was an absence of significant adhesion of both placental and maternal peripheral blood isolates to ICAM-1 whereas 84.2% of child isolates bind to this receptor. The investigators suggested not only a role for CSA as a factor for sequestration, but indicated that low placental blood flow, reduced cell deformability of IRBC, and impaired T-cell responses to infection are also likely to be involved. In a comprehensive review of maternal malaria and parasite adhesion, Fried and Duffy explain the lack of in vivo attachment of IRBCs to villi as resulting from mechanical dislodgment during vaginal delivery. The rhesus monkey malaria-infected placentas were delivered by cesarean section and IRBCs were not observed adhering to the surface of villi. The monkey model will make a valuable contribution toward testing this and many other hypotheses. Whether in vitro studies of cytoadhesion are representative of mechanisms in vivo remains to be seen.
Although there were many similarities between human and monkey placental lesions associated with malaria, there were also differences. It is likely that these differences are attributable to sampling technique. Previous studies of placental pathology in malaria have been primarily descriptive, and few have explored the mechanisms of malaria-induced placental lesions or their relationship to fetal outcome. In most human studies, placental pathology has been performed on a single 1 cm² sample of placental tissue taken from an area closely associated with the basal plate, rather than on full thickness sections. Our study used sampling techniques as described by the College of American Pathologists, which recommends taking full thickness sections from the central area of cotyledons by cutting from the fetal to the maternal surface and including both amnion and decidua. We also examined a minimum of five placental sections taken from five different cotyledons, as well as sections of chorioamniotic membrane and umbilical cord. This technique resulted in more extensive qualitative and quantitative data than has been collected in human studies.

Human studies have often correlated various fetal outcomes with placental malaria. The term “placental malaria”, although widely used, has not been well-defined and has, therefore, produced much confusion. In most studies, placental malaria is defined as the presence of Plasmodium or Plasmodium-infected RBCs within the placenta; others have added malaria pigment as a criterion. Methods used to identify parasites in the placenta include hematoxylin and eosin- Giemsa-stained placental tissue sections, impression smears of cut, uncut, or crushed placental tissue, smears of blood removed with a pipette or needle and syringe, smears of blood drippings from cut placenta allowed to sit for a specified time, smears of blood collected by placental perfusion, and blood taken by undescribed techniques. The term placental malaria may or may not define the infection status of the placenta on the day of delivery, is not indicative of past infections which have cleared, or of chronic infections, and should not be confused with malaria-related histopathology describing specific placental lesions. To improve fetal/infant outcome in pregnant mothers with malaria, the alterations in normal placental morphology and function, and the gestational time when these events occur must be defined. The findings in our study demonstrate the validity of the monkey model and begin to define the pathobiology of malaria during pregnancy. In most human studies, therapeutic interventions cannot be avoided and this further complicates the data. Controlled studies in the monkey model can provide information that will be crucial to successful therapeutic interventions in areas where there are limited resources.

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