1999-11-24

Malaria and pregnancy: placental cytokine expression and its relationship to intrauterine growth retardation

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Et al.

Malaria and Pregnancy: Placental Cytokine Expression and Its Relationship to Intrauterine Growth Retardation

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Malaria infections during pregnancy can lead to the delivery of low–birth-weight infants. In this study, cytokine mRNA was measured in placentas from 23 malaria-infected and 21 uninfected primigravid women who had delivered in Mangochi, Malawi, a region with a high rate of transmission of falciparum malaria. Significantly increased expression of interleukin (IL)–1β, IL-8, and tumor necrosis factor (TNF)–α and decreased expression of IL-6 and transforming growth factor–β1 were found in malaria-infected compared with uninfected placentas. TNF-α and IL-8 were produced by maternally derived hemozoin-laden placental macrophages. Increased TNF-α expression was associated with increased placental hemozoin concentrations. Increased TNF-α or IL-8 expression in the placenta was associated with intrauterine growth retardation but not with preterm delivery. The results suggest that malaria infections induce a potentially harmful proinflammatory response in the placenta.

Malaria can be quite detrimental to pregnant women. In areas with a high rate of malaria transmission, primigravid women are at the greatest risk, whereas in areas with a low rate of malaria transmission, all pregnant women are at risk. Maternal malaria has been associated with delivery of low–birth-weight (LBW) infants because of either preterm delivery (PTD) or intrauterine growth retardation (IUGR) [1–3]. LBW infants have elevated risks of dying during the postneonatal period [4, 5].

Malaria-associated LBW may have different causes in different epidemiologic settings. In areas with a high rate of malaria transmission, infections early in pregnancy are associated with IUGR whereas infections later in pregnancy are associated with PTD [6]. However, the underlying mechanisms responsible for malaria pathogenesis during pregnancy are not well understood.

In this study, we measured cytokine mRNA in placental tissue from primigravid women, with and without malaria, living in an area of Malawi with a very high transmission rate for falciparum malaria. We measured several cytokines suspected of being involved with the pathogenesis of either malaria or poor birth outcome.

Materials and Methods

Study population. Data were collected at Mangochi District Hospital in Mangochi District, an area with a high rate of malaria transmission. Recruitment of subjects and the methods of evaluation are described elsewhere [7]. Placental hemozoin concentrations had been measured in a previous study by using a sensitive fluorometric assay that could detect as little as 20 ng of hemozoin heme per milligram of placental protein [8]. Gestational age was estimated by using the Dubowitz score [9]. Infants delivered before 37 weeks were considered to have had PTDs. Percentile weight for gestational age was assessed by using a standard population and controlling for sex of the infant and gravid category of the mother [10, 11]. The IUGR category consisted of infants in the lowest tenth percentile of weight for gestational age.

Parasitemia of umbilical cord blood is strongly associated with poor birth outcome [6, 7]. All available placental biopsy specimens from women with cord blood parasitemias were used (n = 23). For the control group, we chose placentas from women who showed no signs of malaria infection during pregnancy (n = 21). Although women in the latter group were probably exposed to malaria, they never had detectable antenatal or delivery parasitemias, nor did they have fluorometrically detectable hemozoin in the placenta. Table 1 provides a summary of characteristics of the infected and uninfected groups.

Placental sample collection and isolation of total RNA. Whole placentas were refrigerated until assessment. Within 16 h of delivery, placental samples were collected from an area ~1 inch from the point of insertion of the cord, as described by Bulmer et al. [12]. These samples were rinsed in saline, placed in a cryovial, and

Received 29 March 1999; revised 30 July 1999; electronically published 12 November 1999.

The study protocol was approved by the Malawi Health Sciences Research Committee and the University of Michigan Health Sciences Internal Review Board. Informed consent was obtained from all participants in the study.

Grant support: Conservation, Food, and Health Foundation; International Rotary Foundation; and the National Institutes of Health (D43 TW00908-01).

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The Journal of Infectious Diseases 1999; 180:1987–93

0022-1899/1999/18006-0029$02.00

Table 1. Summary of study population characteristics.

Characteristic	Infected	Uninfected	*P*
No. of subjects	23	21	
Gestational age (w)	37.2 ± 2.0	38.7 ± 1.4	.01
Birth weight (g)	2671 ± 460	3030 ± 417	.01
Term deliveries	10 (43.5)	17 (80.9)	.012
PTD only	7 (30.4)	1 (4.8)	.007
IUGR only	4 (17.4)	3 (14.3)	.28
PTD and IUGR	2 (8.7)	0	.27

NOTE. Data are no. (%) of subjects or mean ± SD. IUGR, intrauterine growth retardation; PTD, preterm delivery.

frozen in liquid nitrogen. Samples were transported in a dry shipper from Malawi to the University of Michigan, where further analysis was conducted. Total RNA was extracted from the placental samples by using the method of Chomczynski and Sacchi [13] with the following modifications. Between 100 and 800 mg of tissue was homogenized in 1 mL of solution D (4 *M* guanidine thiocyanate; 25 m*M* sodium citrate, pH 7.0; 0.5% [wt/vol] sarcosyl) with 0.1 *M* β-mercaptoethanol per 100 mg of tissue by using loose-fitting Dounce tissue grinders (0.0035–0.0055 inch of clearance; Wheaton Scientific, Millville, NJ). The RNA samples were extracted with phenol-chloroform (GibcoBRL, Grand Island, NY) and then precipitated by isopropanol, resuspended, and precipitated by ethanol. The isolated RNA was washed with 80% ethanol and dissolved in high-performance liquid chromotography–grade, ribonuclease (RNase)–free water (Fisher Scientific, Pittsburgh). RNA was stored in aliquots at −80°C until its use.

Ribonuclease protection assay (RPA). RPAs were performed as described elsewhere [14]. Two cytokine-specific riboprobe template sets, HL-14 and HL-23, were assembled from *Eco*RI-linearized and -purified subclones [15]. The HL-14 template set synthesized riboprobes specific for human interleukin (IL)–6, IL-10, IL-1a, tumor necrosis factor (TNF)–β, granulocyte-macrophage colony–stimulating factor (GM-CSF), transforming growth factor (TGF)–β1, IL-1β, TNF-α, and rpL32. The HL-23 template set synthesized riboprobes specific for human IL-12p40, IL-4, IL-5, interferon (IFN)–γ, IL-2, IL-13, IL-12p35, and rpL32. In this study, IL-8 replaced IL-13 in the HL-23 template set. The RPA riboprobe subclones were generated as described elsewhere [15] with the addition of IL-8 (nucleotides 2467–2598; GenBank accession no. M28130). All riboprobe syntheses were driven by T7 bacteriophage RNA polymerase (Promega, Madison, WI) with [α-^{32}P] UTP (3000 Ci/mmol) (Amersham, Arlington Heights, IL) as the labeling nucleotide in an in vitro transcription reaction. The subsequent steps of probe purification, total placental RNA (15 μg)–probe hybridization, RNase treatment, purification of protected RNA duplexes, and resolution of protected probes by standard 5% acrylamide/8-*M* urea sequencing gels were performed exactly as described by Hobbs et al. [14]. Probe bands were visualized by autoradiography (XAR film; Kodak, Rochester, NY) and were quantified with Storm PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA). For the latter task, volume measurements with rectangular objects were used to generate PhosphorImager (PI) counts. PI counts were normalized to account for varying numbers of incorporated [α-^{32}P] UTPs for each probe, as well as for lane-to-lane and assay-to-assay variability, and each PI count is presented as a fraction of the internal housekeeping signal (rpL32) in each lane.

Reverse transcription–polymerase chain reaction (RT-PCR). Total RNA from each placental sample was used as the template for RT-PCR with the GeneAmp RNA PCR kit (PE Biosystems, Foster City, CA) according to the manufacturer's instructions. For amplification of human immunodeficiency virus (HIV) sequences, *gag*-specific primers SK38 and SK39 [16] were used. IL-1α was amplified as a positive control by using primers DM151 and DM152 supplied in the GeneAmp kit. The PCR mix was initially heated at 95°C for 3 min, with the PCR primers added at 30 s. PCR was then done for 43 cycles of 30 s at 94°C and 1 min at 60°C, followed by a final extension of 7 min at 72°C in a PTC-100 thermocycler (MJ Research, Watertown, MA).

For a positive control, HIV cDNA was provided by Dr. Stephen King at the University of Michigan in Ann Arbor. HIV cDNA was further diluted into placental cDNA (derived from the RT of 1 μg total RNA) to a concentration of 100 copies of HIV per reaction. The amplified PCR products were analyzed by 2% agarose gel electrophoresis with ethidium bromide (5 μg/mL) staining. The signal intensities of the bands were scored as either greater or less than 100 copies of HIV per microgram of total RNA.

Antibodies and immunohistochemistry (IHC). Mouse monoclonal antibodies were purchased from Pharmingen (San Diego). Optimal concentrations were 20 μg/mL for anti-human TNF-α (clone mAb11) and 10 μg/mL for anti-human IL-8 (clone G265-8). Purified mouse IgG1, κ-isotype standard (clone MOPC-21), served as the negative control. Acetone-fixed frozen sections were stored at −80°C until stained. Standard indirect immunostaining was performed with solutions containing 0.1% saponin. PBS-rehydrated sections were serially pretreated with 1% H_2O_2, avidin-biotin, and a 1 : 50 dilution of normal goat serum (BioGenex, San Ramon, CA). Then optimal dilutions of cytokine-specific and control antibodies were applied for 1–2 h at room temperature. After washing, 10 μg/mL biotin-conjugated goat anti-mouse IgG was applied for 30 min. The slides were washed in saponin-free PBS, overlaid with ABC reagent (Vector Laboratories, Burlingame, CA) for 15 min, washed again, and then incubated with the substrate (3-amino-9-ethyl carbazole [AEC]) for 2–5 min. The substrate was rinsed away with water, and Mayer's hematoxylin was used as a counterstain for viewing.

Statistical analyses. Methods used included *F* tests for 2-sample variances, 2-tailed *t* tests for significant differences in means, and linear regression done with Microsoft Excel 97 (Redmond, WA). A 1-tailed Fisher's exact test was done with Epi Info 6.04b (Centers for Disease Control and Prevention, Atlanta, GA) to assess relationships between pairs of variables. Bonferroni's adjustment was used as a more conservative measure of significance (α = 5%) for multiple comparisons [17].

Results

Placental cytokine expression pattern associated with malaria infection. We measured RNA levels for a number of cytokines in malaria-infected and -uninfected placentas. None of the placentas contained RNA for IFN-γ, IL-2, IL-4, IL-5, IL-12, or GM-CSF (data not shown). RNA for IL-1α, TNF-β, and IL-10 was detected, but there were no significant differences between infected and uninfected placentas (table 2). In contrast, we found significant ($P < .05$) differences in mRNA levels be-

Table 2. Placental cytokine mRNA levels from women with and without malaria.

Cytokine	Infected (mean ± SD)[a]	Uninfected (mean ± SD)[a]	Infected/uninfected	*P*
IL-1α	0.097 ± 0.092	0.090 ± 0.086	1.08	.80
IL-1β	1.01 ± 1.05	0.328 ± 0.41	3.08	.007
IL-6	0.007 ± 0.006	0.011 ± 0.008	0.60	.04
IL-8	0.763 ± 0.768	0.358 ± 0.241	2.13	.02
IL-10	0.017 ± 0.032	0.014 ± 0.010	1.21	.76
TGF-β1	2.595 ± 1.106	5.288 ± 4.143	0.49	.005[b]
TNF-α	0.475 ± 0.511	0.123 ± 0.083	3.86	.004[b]
TNF-β	0.032 ± 0.021	0.037 ± 0.022	0.86	.50

NOTE. IL, interleukin; TGF, transforming growth factor; TNF, tumor necrosis factor.

[a] Ratio of volume of cytokine signal to that of rpL32, a housekeeping gene, in same lane on the gel.

[b] Significant ($\alpha = 5\%$) after Bonferroni's adjustment for multiple comparisons.

tween infected and uninfected placentas for IL-6, TGF-β1, IL-1β, IL-8, and TNF-α (table 2). In the infected placentas, IL-6 and TGF-β1 transcripts decreased by 40% and 51%, respectively, whereas IL-1β, IL-8, and TNF-α increased 3-fold, 2-fold, and almost 4-fold, respectively. Using Bonferroni's adjustment for multiple comparisons, the differences in TGF-β and TNF-α expression remained significant, and IL-1β was borderline significant.

Degradation not the cause of differences in cytokine RNA levels. Varying periods of time elapsed between delivery and processing of the placenta (15 min to 16 h). Regression analyses were performed to determine whether the elapsed time affected cytokine mRNA levels. The average times before freezing were 7.07 ± 4.01 h for infected samples and 4.96 ± 4.96 h for uninfected samples. No significant differences were found (data not shown).

HIV screening of placental RNA by RT-PCR. In general, trophoblastic cells of HIV-positive placentas express significantly higher levels of IL-1β, IL-6, and TNF-α compared with those of uninfected placentas [18]. Therefore, RT-PCR was performed on placental RNA to screen samples for HIV. The lowest level of detection by this method was 100 copies of HIV present in 1 μg of total placental RNA. None of the 44 samples evaluated in this study had HIV levels >100 copies in 1 μg of total RNA. IL-1α expression was used as a positive control for RT-PCR and was successfully detected in all samples (data not shown).

Placental hemozoin concentration associated with TNF-α expression. Placental hemozoin is a good marker of past or current malaria infection [12]. Hemozoin concentrations from the placentas used in this study had been assayed in a previous study by using a fluorometric assay [8]. We performed regression analysis on the infected samples to determine whether hemozoin concentrations were correlated with placental cytokine expression. Of all of the cytokines listed in Table 2, only TNF-α showed a positive association with hemozoin in the placenta ($R^2 = .47$, $P < .0003$), which was significant after Bonferroni's adjustment for multiple comparisons).

Detection of IL-8 and TNF-α in placental tissue sections by IHC. Using IHC, we verified that IL-8 and TNF-α transcripts were being translated, and we identified which placental cell types were involved in their expression. In uninfected placental samples, anti–IL-8 monoclonal antibodies stained the fetal vascular endothelial cells within the chorionic villi (figure 1*A*). Malaria-infected placental sections incubated with anti–IL-8 monoclonal antibodies also showed staining of the fetal vascular endothelial cells within the chorionic villi (figure 1*B*). In addition, some hemozoin-laden placental macrophages stained positively for IL-8. Placental sections from malaria-infected samples stained with mouse IgG control antibodies were negative (figure 1*C*). When malaria-infected placental samples were incubated with monoclonal antibodies against TNF-α, we found staining localized to some hemozoin-laden macrophages (figure 2*A*). Staining was absent in most hemozoin-laden macrophages, in macrophages without hemozoin, and in other cell types. Malaria-infected placental samples incubated with mouse IgG control antibodies were negative (figure 2*B*). Uninfected placental samples stained with anti–TNF-α were negative (data not shown). Slides stained with anti–TNF-α antibodies showed a punctate nonspecific staining pattern throughout that was similar to the pattern seen on slides stained with the IgG control antibody.

Elevated placental expression of either TNF-α or IL-8 associated with IUGR. In the larger study population, 36.6% of deliveries were associated with poor birth outcomes [7]. Accordingly, we dichotomized TNF-α and IL-8 expression levels in infected placenta, so that women whose levels were in the top 35% were classified as high expressers and the others were classified as low expressers. We counted birth outcomes as normal, IUGR, or PTD. Those that were both IUGR and PTD were included in both analyses. Fisher's exact test showed that elevated levels of TNF-α or IL-8 expression were not associated with PTD ($P = .63$ and $P = .37$, respectively). In general, elevated TNF-α or IL-8 had a weak association, or no association, with poor birth outcomes (combined PTD and IUGR; $P = .63$ and $P = .04$, respectively). However, IUGR was significantly associated with elevations in either TNF-α or IL-8 levels ($P = .0086$ for both).

Discussion

Placentas with evidence of past or present malaria infections produce a different blend of cytokines than placentas without such evidence. Infected placentas had 2–4 times the amount of IL-1β, IL-8, and TNF-α mRNA, and about half as much IL-6 and TGF-β1 RNA as uninfected placentas. Among the infected placentas, higher hemozoin content was associated with a greater amount of TNF-α mRNA. IHC showed that hemozoin-laden macrophages produce both TNF-α and IL-8. Increased expression of either TNF-α or IL-8 appears to be associated with IUGR. The association seen here between either TNF-α or IL-8 and IUGR is provisional because it was found

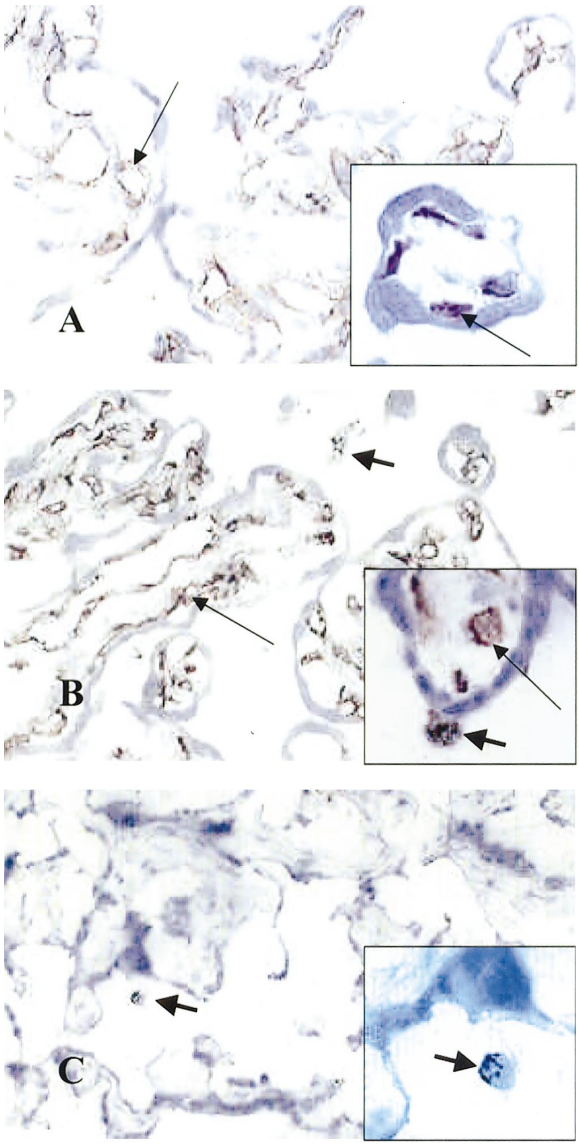

Figure 1. Placental tissue sections stained with mouse monoclonal anti–interleukin (IL)-8 antibodies from uninfected sample *(A)* and malaria-infected sample *(B)*; IgG control antibody staining of malaria-infected placental sample *(C)* with streptavidin-biotin immunostain and hematoxylin counterstain. Long arrows indicate IL-8 staining of fetal endothelial cells in chorionic villi in both uninfected and infected placental tissue. Bold arrows indicate hemozoin-laden macrophages that were positive for IL-8 staining in infected tissue or were not stained in IgG control sample. Insets provide magnified views of hemozoin-laden macrophages and staining patterns. Original magnification, ×1000 (oil immersion).

in a relatively small sample. However, the *P* values were quite robust, and the data are consistent with those of previous studies on both malarial [19] and nonmalarial [20] causes of LBW.

Our observations of increased proinflammatory cytokines (TNF-α and IL-1β) and decreased anti-inflammatory cytokines (IL-6 and TGF-β) are consistent with the results of many other studies, reviewed by Clark et al. [21], that show that severe malaria infections tend to be associated with increased proinflammatory cytokines in the peripheral circulation. The only exception is an increase in IL-6, which has been associated with increased severity of malaria [22], even though IL-6 is anti-inflammatory [23]. Thus, the role of IL-6 in pathogenesis needs further clarification.

Previous studies have also shown proinflammatory responses in placentas from malaria-infected women [19, 24]. In a study by Fried et al. [19], placentas from infected primigravid women had more TNF-α, about the same amount of IL-10, and less IL-6 compared with placentas from uninfected women. Placental TNF-α was associated with poor birth outcome. These results are almost identical to those reported here.

The major difference between our study and that of Fried et al. [19] is that we measured cytokine mRNA, whereas the latter group measured cytokine protein. For the cytokines IFN-γ, IL-2, and IL-4, Fried et al. detected the protein in placentas, whereas we did not detect the corresponding RNA. Also, Fried et al. detected slightly more placental TGF-β in infected women than in uninfected women, whereas we detected significantly less TGF-β RNA in infected women. Two possible explanations for differences between measurements of placental cytokine protein and RNA are as follows. First, some cytokine protein present in the placenta might have been synthesized outside of the placenta. Second, it is possible that the mRNA for some cytokines is especially labile and might deteriorate during storage.

Unlike the other cytokines measured in this study, IL-8 is a chemokine. Our finding of increased IL-8 in malaria-infected placentas is consistent with the results of a previous study that showed that malaria infections are associated with elevated levels of IL-8 in the peripheral circulation [25].

Our study is the first to determine which placental cells are responsible for malaria-associated cytokine production. Both TNF-α and IL-8 were made by only some hemozoin-laden placental macrophages. The presence of hemozoin in macrophages suggests that the cell had previously phagocytosed a malaria-infected erythrocyte or parasite-derived debris. The induction of TNF-α production in macrophages by the phagocytosis of parasites is consistent with the in vitro observations of Xiao et al. [26]. It is predictable that TNF-α would be expressed in only a minority of these macrophages, because TNF-α can be produced for only a limited time after the action of a phagocytic stimulus. Also, the production of IL-8 by fetal endothelial cells is consistent with the results of previous studies in which other cytokines were found to be produced by this cell type in uninfected placentas [27].

Selection bias may have affected our results. In particular, because HIV-infected pregnant women are more likely to have parasitemias than women who are not HIV infected [28, 29], the malaria-infected group could have had a higher prevalence

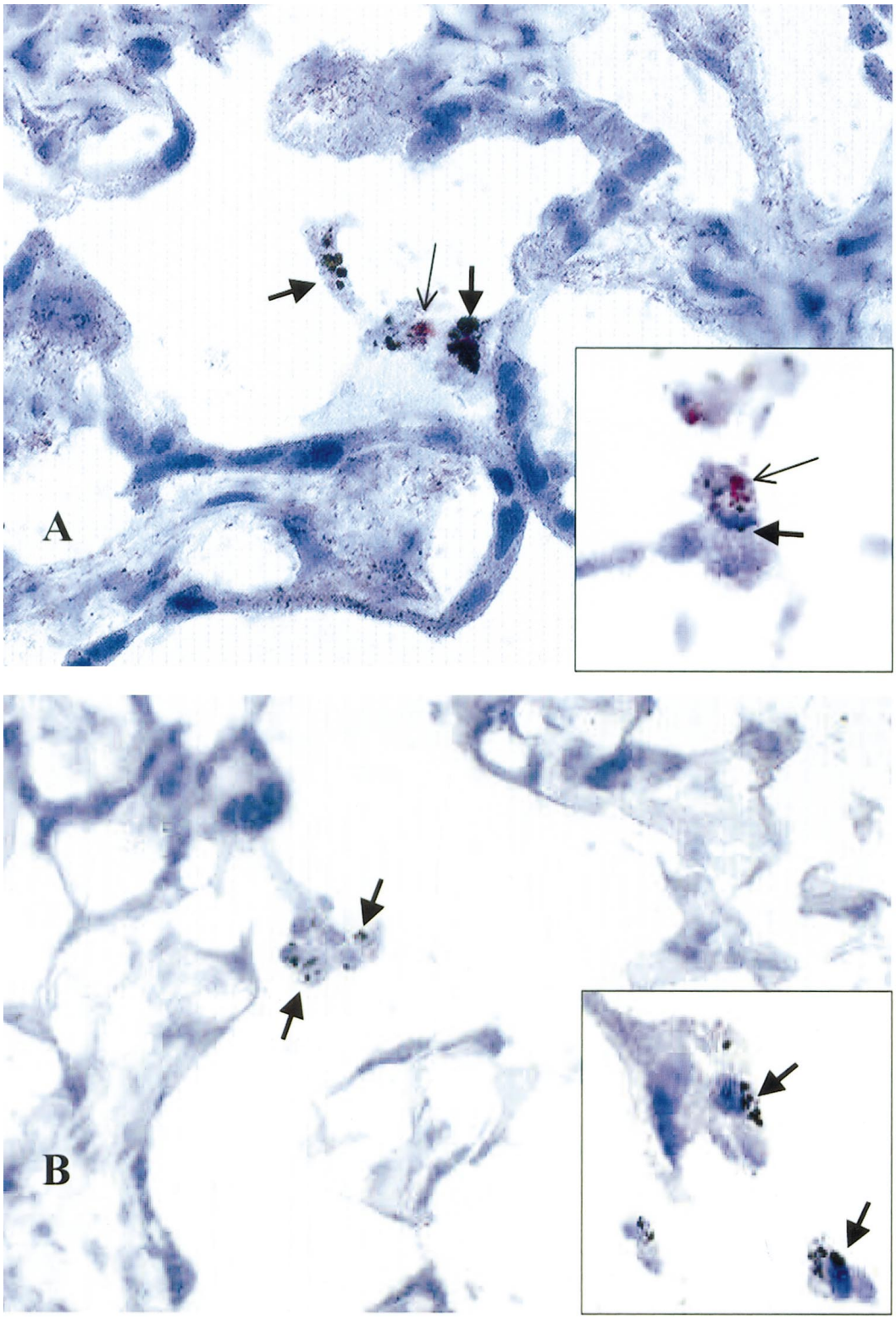

Figure 2. Malaria-infected placental tissue section stained with mouse monoclonal anti–tumor necrosis factor (TNF)–α antibodies *(A)* and IgG control *(B)*, and streptavidin-biotin immunostain with hematoxylin counterstain. Long arrows indicate TNF-α staining in hemozoin-laden macrophages. Bold arrows indicate hemozoin pigment in macrophages. Insets provide magnified views of hemozoin-laden macrophages and staining pattern. Original magnification, ×1000 (oil immersion).

of HIV infection. Even though no HIV virus was detected in any placenta used in this study, some of the mothers may have been infected with HIV. Given that both placental monocytes and trophoblasts from HIV-infected women produce increased amounts of proinflammatory cytokines [18], HIV could have mediated the effect measured in this study. However, HIV status does not seem to affect the prevalence of cord blood parasitemia [29]. Also, TNF-α production appears to depend on the presence of PCR-detectable virus [18], and no PCR-detectable viruses were found in the placentas we studied. Thus, this confounding effect is not likely. In addition, the fact that TNF-α and IL-8 were visualized in macrophages containing parasite remnants (hemozoin) suggests that malaria directly induces these cytokines.

Malaria-induced alterations in cytokine secretion might mediate the effects of malaria on a first pregnancy. TNF-α and IL-1 play a role in normal fetal development and parturition [30, 31]. IL-1β and IL-8 appear to be responsible for normal cervical ripening [32]. Increased placental TNF-α, IL-1, and IL-8 have been associated with PTD that is due to other causes [32–39]. TNF-α and IL-1β may have this effect by increasing prostaglandin synthesis and efficacy [40–42]. Increased placental TNF-α may cause IUGR [20] by preventing nutrient uptake by the fetus [43].

The importance of cytokines in mediating the effects of malaria on pregnancy is consistent with the observation that *Plasmodium vivax* infections also adversely affect birth outcome [44]. *P. vivax* infections are associated with greater increases in TNF-α than *P. falciparum* infections [45].

In summary, malaria infections in primigravid women appear to cause significant alterations in the pattern of placental cytokine synthesis. These alterations might be responsible for some of the LBW observed in the offspring.

Acknowledgments

We would like to thank Martin Philbert, Tony Schork, and Judy Poore for guidance and assistance; Stephen King and Gary Nabel for providing HIV cDNA. This article is dedicated to the memory of Monte Hobbs (whose devotion to technical excellence made the cytokine RPAs possible).

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