Distinct Clinical and Immunologic Profiles in Severe Malarial Anemia and Cerebral Malaria in Zambia

Philip E. Thuma,[1] Janneke van Dijk,[1] Rick Bucala,[2] Zufan Debebe,[3] Sergei Nekhai,[3] Thea Kuddo,[3] Mehdi Nouraie,[3] Günter Weiss,[4] and Victor R. Gordeuk[3]

[1]Malaria Institute at Macha, Choma, Zambia; [2]School of Medicine, Yale University, New Haven, Connecticut; [3]Center for Sickle Cell Disease, Howard University, Washington, D.C; and [4]Department of Internal Medicine, Medical University of Innsbruck, Innsbruck, Austria.

Background. The mechanisms of severe malarial anemia and cerebral malaria, which are extreme manifestations of *Plasmodium falciparum* malaria, are not fully understood.

Methods. Children aged <6 years from southern Zambia presenting to the hospital with severe malarial anemia ($n = 72$), cerebral malaria ($n = 28$), or uncomplicated malaria ($n = 66$) were studied prospectively. Children with overlapping severe anemia and cerebral malaria were excluded.

Results. Low interleukin 10 concentrations had the strongest association with severe anemia (standard $\beta = .61$; $P < .001$) followed by high tumor necrosis factor α and sFas concentrations, low weight-for-age *z* scores, presence of stool parasites, and splenomegaly (standard $\beta = .15–.25$; $P \leq .031$); most of these factors were also associated with lower reticulocytes. Greater parasitemia was associated with higher interleukin 10 and tumor necrosis factor α concentrations, whereas sulfadoxizole/pyrimethamine therapy and lower weight-for-age *z* scores were associated with lower interleukin 10 levels. Thrombocytopenia and elevated tissue plasminogen activator inhibitor 1 levels had the strongest associations with cerebral malaria (standard $\beta = .37$ or .36; $P < .0001$), followed by exposure to traditional herbal medicine and hemoglobinuria (standard $\beta = .21–.31$; $P \leq .006$).

Conclusions. Predictors of severe malarial anemia (altered immune responses, poor nutrition, intestinal parasites, and impaired erythropoiesis) differed from those of cerebral malaria (thrombocytopenia, herbal medicine, and intravascular hemolysis). Improved preventive and therapeutic measures may need to consider these differences.

Severe malarial anemia and cerebral malaria are life-threatening presentations of *Plasmodium falciparum* malaria that are especially prevalent in African children and for which the mechanisms are not fully known. Both acceleration of erythrocyte destruction and inhibition of erythrocyte production contribute to anemia associated with malaria [1]. In severe anemia, bone marrow abnormalities include ineffective erythropoiesis, dyserythropoiesis, and lower erythroblast proliferative rates [2]. Dysregulation of host immunologic pathways, such as an excessive or sustained innate immune response [3], a polarization of adaptive T-cell responses toward the production of mediators that suppress normal pathways of erythropoietic development [4], excessive production of macrophage migratory inhibitory factor [5], and low circulating levels of interleukin (IL)-10 [6] may contribute to suppression of erythropoiesis.

Immune dysregulation also has been proposed in the pathogenesis of cerebral malaria. For example, elevated circulating concentrations of IL-6, IL-10, tumor necrosis factor (TNF)-α, or other markers have been associated

Received 20 May 2010; accepted 2 September 2010.

Potential conflicts of interest: none reported.

Presented in part: Annual meeting of The American Society of Tropical Medicine and Hygiene, Atlanta, GA, 12–16 November, 2006. Poster #853.

Reprints or correspondence: Dr Victor R. Gordeuk, Center for Sickle Cell Disease, Howard University, 2041 Georgia Ave NW, Washington, DC 20060 (vgordeuk@howard.edu).

The Journal of Infectious Diseases 2011;203:211–219

1537-6613/2011/2032-0001$15.00
DOI: 10.1093/infdis/jiq041

with cerebral malaria in African children [7, 8]. Histopathologic studies have indicated sequestration of parasitized red cells and vascular endothelial activation in patients with fatal cerebral malaria, and cerebral microvascular thrombi or fibrin deposition may be observed as well [9–11]. Endothelial dysfunction related to the degree of hemolysis and to decreased nitric oxide availability is another potential etiologic pathway for cerebral malaria [12].

In this study, we investigated the possible roles of clinical, environmental, and immunologic mechanisms in the pathogenesis of severe malaria by prospectively studying Zambian children who presented with uncomplicated malaria, severe malarial anemia, or cerebral malaria.

METHODS

Study of Children with Malaria

This prospective study was designed to determine whether immune dysregulation, manifested as an excessive helper T (T_H-)1 and proinflammatory immune response and a decreased IL-10 response, may be a major contributing factor in the severe anemia that develops in some children infected with *P. falciparum*. The study design called for the study of severe malarial anemia patients in parallel with the study of both uncomplicated malaria patients and cerebral malaria patients. Patients with overlapping severe malarial anemia and cerebral malaria were excluded.

The study was conducted prospectively among children of the Tonga ethnic group admitted to Macha Mission Hospital in southern Zambia with the clinical diagnosis of malaria from March 2001 through May 2005. The study was approved by the Ethical and Research Committee of the University of Zambia (Lusaka, Zambia) and the Committee on Human Investigation of Howard University (Washington, DC). Written informed consent was obtained for each participant. Inclusion criteria included age <6 years and the presence of asexual forms of *P. falciparum* determined by light microscopic examination (1000x) of a thick smear of the peripheral blood stained with Giemsa. A standard scoring system was used in which "0" indicated no parasites found in 100 fields, "1+" indicated 1–10 parasites per 100 microscopic fields, 2+ indicated 11–100 parasites per 100 microscopic fileds, 3+ indicated 1–10 parasites in one microscopic field and 4+ indicated >10 parasites in one microscopic field. Children were recruited prospectively into 1 of 3 groups—severe malarial anemia, cerebral malaria, or acute uncomplicated malaria—with the goal of having the same number of participants in each group. Because of a declining incidence of cerebral malaria during the study, we were unable to recruit as many patients with cerebral malaria as patients with severe malarial anemia or uncomplicated malaria.

Children with acute uncomplicated malaria had a screening hematocrit level of ⩾18% as performed by capillary tube centrifugation on a finger-stick blood specimen and the absence of coma as defined by a Blantyre coma score of 5 [13]. Children with severe malarial anemia had a screening hematocrit level of <15% and the absence of coma. Children with cerebral malaria had a screening hematocrit level of ⩾18% and unarousable coma as defined by a Blantyre coma score of ⩽2 that persisted for >30 minutes after a seizure if 1 was observed and for which there was no other identifiable cause such as hypoglycemia or meningitis. Venipuncture blood samples were obtained for both routine clinical care as well as for determination of immunological markers before the start of antimalarial treatment. We stored plasma samples at −20°C to −70°C until analysis. We made a complete blood count with an automated cell counter (Cobas Micros CT, Roche Diagnostics). We determined the percentage of reticulocytes by microscopic counting of wet preparations of peripheral blood stained with a 1% methyl alcohol solution of cresyl blue. We examined urine microscopically and by dipstick method for heme (Urispec 11-way, Henry Schein). We examined stool specimens microscopically for ova and parasites. We did not have permission from the Ethical and Research Committee of the University of Zambia to routinely perform HIV testing as a part of this study.

Analysis of Immune Markers

A panel of 26 immune biomarkers was measured by a multiplex analysis system (Biorad Laboratories), which is a bead-based, flow cytometric assay that permits the simultaneous analysis of different biomolecules in a single microplate well. Plasma concentrations were measured in duplicate using Human Cytokine/Chemokine Multiplex Immunoassay kits (LINCO Research) for IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-15, IL-17, interferon (IFN)-γ, granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, TNF-α, eotaxin, monocyte chemoattractant protein 1, macrophage inflammatory protein 1α, and interferon-inducible protein 10 and Human Sepsis/Apoptosis Multiplex Immunoassay kits (LINCO Research) for total plasminogen activator inhibitor 1 (PAI-1), sFas ligand, sFas, and macrophage migration inhibitory factor. In general, high plasma TNF-α concentrations may not reflect bioactive cytokine, but instead levels of nonbioactive TNF-α complexed to its binding protein, soluble TNF receptor 1. However, plasma TNF-α concentrations correlated strongly with concentrations of IL-6 ($R = .67$; $P < .0000001$) and IL-1α ($R = .47$; $P < .0000001$) among the children in this study, suggesting that the plasma levels of TNF-α likely did reflect bioactivity.

The original study protocol called for determination of IL-12, IFN-γ, TNF-α, macrophage migration inhibitory factor, IL-4, and IL-10 because of reported associations of these markers with severe malaria [1, 4, 6, 7, 14, 15] and their relevance to the study hypotheses. The additional immunological markers also were determined based on the availability of appropriate assays for

a small quantity of plasma and potential relevance to malarial pathways: IL-1, IL-2, IL-6, IL-7, IL-8, IL-13, IL-15, IL-17, granulocyte colony-stimulating factor, granulocyte monocyte colony-stimulating factor, interferon-inducible protein 10, monocyte chemoattractant protein 1, macrophage inflammatory protein 1α, sFas, sFas ligand, and PAI-1 because of possible associations with acute or severe malaria or erythropoiesis [7, 16–24], and IL-5 and eotaxin because of a possible relationship between eosinophil response and malarial anemia [25].

Statistics

Continuous variables were compared with the Kruskal-Wallis test, and categorical variables were compared with the Fisher exact test. Relationships between 2 continuous variables were assessed with the Spearman correlation coefficient. Pathway analysis was used to assess the multivariate relationships of severe malarial anemia and cerebral malaria with clinical characteristics and with immune markers. In these models of severe malarial anemia or cerebral malaria, uncomplicated malaria served as the reference group. Pathway analysis is a multivariate statistical technique used to examine causal relationships among 2 or more variables. In this analysis a hypothetical pathway from exogenous variables (predictors) to endogenous variables (responses) is tested against observed data. A standardized regression weight is computed for each causal relationship as adjusted for the other relationships. If the data supports the model, the overall P value will not be significant. The fitness of final model to the observed data can be measured by several indicators, including the root mean square error of approximation (RMSEA), which measures the discrepancy function obtained by fitting the model to the population values. A RMSEA of $\leq .05$ indicates a close fit of the model. Analyses were performed with STATA (version 10.1; StataCorp) or AMOS (version 18.0; SPSS).

RESULTS

Children with Severe Malarial Anemia Compared with Children with Uncomplicated Malaria

Clinical Characteristics. We found that 72 children with severe malarial anemia were of similar age and sex as 66 children with acute uncomplicated malaria, but they were almost twice as likely to have evidence of malnutrition based on a weight-for-age z score of < -2.0 (Table 1). The median duration of febrile illness before presentation was more than twice as long in the children with severe anemia, and greater proportions of these children had been treated before presentation with traditional medicine or sulfadoxine/pyrimethamine; the latter antimalarial drug is known to have decreasing efficacy for *P. falciparum* in this part of Africa [26]. The children with severe anemia had lower temperatures, less parasitemia, and more hepatomegaly than did children with uncomplicated malaria. Mean corpuscular volumes, platelet counts, and white blood cell counts were higher in children with severe malarial anemia, whereas the prevalence of heme positive urine did not differ significantly from that of children with uncomplicated malaria.

Immune Markers. The median plasma IL-10 concentration in children with severe anemia was less than one-fifth the level in children with uncomplicated malaria, but the other originally selected markers did not differ significantly between the groups in bivariate analysis (Table 2a). Of the additional immune markers determined, IL-1α and interferon-inducible protein 10 concentrations were significantly lower in patients with severe malarial anemia than in patients with acute uncomplicated malaria, and concentrations of soluble Fas and PAI-1 were significantly higher (Table 2b).

Pathway Analysis of Severe Anemia. In a pathway analysis of the clinical data and originally chosen immune markers, higher IL-10 levels ($\beta = -.66$; $P < .001$) and higher weight-for-age z scores ($\beta = -.18$; $P = .008$) were associated with a decreased risk of severe malarial anemia, whereas higher TNF-α levels ($\beta = .26$; $P = .003$), the presence of stool parasites ($\beta = .16$; $P = .021$), and splenomegaly ($\beta = .17$; $P = .006$) were associated with increased risk. Higher weight-for-age z scores were also associated with higher parasite counts ($\beta = .25$; $P < .001$) and higher IL-10 concentrations ($\beta = .14$; $P = .014$). In turn, greater parasitemia was associated with higher concentrations of both IL-10 ($\beta = .35$; $P < .001$) and TNF-α ($\beta = .34$; $P < .001$). A history of sulfadoxizole/pyrimethamine therapy was associated with decreased parasitemia ($\beta = -.37$; $P < .001$) and, in keeping with a previous report in a different setting [27], lower IL-10 levels ($\beta = -.21$; $P < .001$). There were significant nondirectional associations between severe anemia and malaria parasitemia (partial $r = -.22$; $P = .005$) and between weight-for-age z scores and the presence of stool parasites ($\beta = -.22$; $P = .016$). The model predicted 49% of variation in the risk of severe anemia and 20% of the variation in parasite count. The model had a good fit to data ($\chi^2 = 14.8$; $df = 14$; $P = .4$), and it was statistically significant (RMSEA = .02; 90% CI, 0.0–.07).

In an analysis that included both the original and additional immune markers, only sFas proved to make a significant and consistent addition to the model. Higher sFas levels ($\beta = .22$; $P = .001$) were associated with severe anemia and lower parasite count.

These findings in pathway analysis were confirmed in a logistic regression model. In this model, a decrease in the IL-10 concentration by 1 standard deviation (SD) was associated with an estimated 11.1-fold increase in the odds of severe anemia ($P < .001$), an increase in the TNF-α concentration by 1 SD with a 4.7-fold increase in the odds ($P = .001$), an increase in the sFas concentration by 1 SD with a 4.4-fold increase in the odds ($P = .001$), a 1 cm increase in spleen size by palpation with a 2.2-fold increase in the odds ($P = .003$), a 1-point decrease in the weight-for-age z score with a 2.4-fold increase in the odds

Table 1. Clinical Characteristics of Children with Malaria by Category

	N	Uncomplicated Malaria	*N*	Severe Malarial Anemia	*N*	Cerebral Malaria	*P* (Severe Anemia vs Uncomp.)	*P* (Cerebral vs Uncomp.)	*P* (Severe Anemia vs Cerebral)
Demographic and historical characteristics									
Age, mon	66	26 (18–36)	72	22 (16–31)	28	37 (26–43)	.059	.005**	<.0005**
Female sex*	66	33 (50.0%)	72	40 (55.6%)	28	11 (39.3%)	.609	.4	.14
Fever duration, h	66	44 (27–81)	72	104 (74–151)	28	48 (31–73)	<.0005**	>.9	.4
Seizures*	66	17 (25.8%)	72	24 (33.3%)	28	25 (89.3%)	.4	<.0005**	<.0005**
Treatment with traditional medicine	66	14 (21.2%)	72	42 (58.3%)	28	14 (50.0%)	<.0005**	.007**	.5
Antimalarial treatment									
Chloroquine*	64	14 (21.9%)	71	30 (42.3%)	27	12 (44.4%)	.016	.042	.8
Sulfadox/pyrimethamine.*	64	12 (18.8%)	70	33 (47.1%)	28	9 (32.1%)	.001**	.2	.18
Chloroquine and/or sulfadox./pyr*.	66	23 (34.9%)	72	53 (73.6%)	28	19 (67.9%)	<.0005**	.006**	.6
Quinine*	63	0 (0%)	69	3 (4.3%)	25	3 (12.0%)	.2	.021	.18
Clinical findings at presentation									
Weight, kg	65	10.7 (8.8–12.4)	71	9.1 (7.9–10.7)	28	12.1 (10.9–12.9)	<.0005**	.010	<.0005**
Weight-for-age *z* score $\leqslant -2$*	65	25 (38.5%)	71	50 (70.4%)	28	9 (32.1%)	<.0005**	.6	<.0005**
Temperature, °C	66	37.8 (36.6-38.6)	72	36.8 (36.3–37.6)	28	37.8 (37.3–38.3)	.001**	.4	<.0005**
Deep breathing*	66	0 (0%)	72	4 (5.6%)	28	5 (17.9%)	.1	.002**	.054
Liver size, cm below costal margin	65	0 (0–1)	71	2 (0–3)	26	2 (1–3)	<.0005**	<.0005**	.5
Spleen, cm below costal margin	66	1 (0–2)	72	2 (0.5–2)	26	1.5 (0–2)	.032	.7	.2
Hematocrit, %	66	29.5 (24.0–33.0)	72	12.0 (9.0–13.0)	28	23.0 (20.5–30.0)	—	.027	<.0005**
Mean corpuscular volume, fL	65	70 (63–76)	68	76 (68–83)	27	72 (68–79)	.001**	.1	.3
Reticulocytes, X $10^{-3}/\mu L$	63	45 (26–70)	66	20 (9–45)	27	41 (24–83)	<.0005**	.9	.002**
White blood cells, X $10^{-3}/\mu L$	65	7.9 (5.8–11.0)	69	10.4 (8.0–16.4)	28	8.3 (6.4–11.4)	<.0005**	.4	.018
Absolute lymphocyte count	65	2.4 (1.4–3.5)	68	4.4 (3.0–6.7)	28	2.1 (1.4–3.1)	<.0005**	.4	<.0005**
Platelets, X $10^{-3}/\mu L$	65	114 (76–169)	69	153 (102–218)	28	67 (39–139)	.006**	.017	<.0005**
Malaria parasites, X $10^{-3}/\mu L$	66	147 (26–254)	70	95 (0.1–153)	27	99 (11–302)	<.0005**	.6	.01
Heme-positive urine*	57	2 (3.5%)	60	5 (8.3%)	25	14 (56.0%)	.4	<.0005**	<.0005**
Stool parasites present*	48	1 (2.1%)	56	7 (12.5%)	18	0 (0%)	.066	>.9	.18

NOTE. Results are given as median (interquartile range), unless otherwise indicated.

*Number (%).

**The *P* value remains significant after adjustment for multiple comparisons.

($P = .004$), and the presence of stool parasites with an 18.1-fold increase in the odds ($P = .046$).

We substituted reticulocyte count for severe malarial anemia in the pathway models and found that lower reticulocyte count could replace severe malarial anemia and produce an almost identical model (Table 3), except that spleen size and sFas did not have significant effects on reticulocyte count. This is evidence that high TNF-α, low IL-10, low weight-for-age *z* score, and the presence of stool parasites may be mechanistically related to severe malarial anemia by their associations with a suppression of erythropoiesis.

Children with Cerebral Malaria Compared with Children with Uncomplicated Malaria

Clinical Characteristics. The children with cerebral malaria were older than the children with acute uncomplicated malaria, had more frequently experienced seizures, and were more than twice as likely to have been given traditional herbal medicine (Table 1). Deep breathing and hepatomegaly were significantly more common in the children with cerebral malaria. Urine tested positive for heme in more than half of the children with cerebral malaria and in <5% of the children with uncomplicated malaria. Microscopic examination of the urine revealed the absence of red blood cells. Parasitemia did not differ significantly between patients with cerebral malaria and those with uncomplicated malaria.

Immunological Markers. In contrast to those of the children with severe malarial anemia, none of the plasma concentrations of the originally selected immune markers differed significantly between children with cerebral malaria and those with acute uncomplicated malaria in bivariate analysis (Table 2a). Of the additional immune markers, only tissue PAI-1

Table 2. Plasma Concentrations of Cytokines and Other Immune Molecules According to Participant Group

	Uncomplicated Malaria (n = 56)	Severe Malarial Anemia (N = 56)	Cerebral Malaria (N = 23)	*P* (Severe Anemia vs Uncomp.)	*P* (Cerebral vs Uncomp.)	*P* (Severe Anemia vs Cerebral)
a. Original markers						
Interleukin-12 detectable*	8 (14.3%)	3 (5.4%)	4 (17.4%)	.1	.6	.6
Interferon-γ detectable*	13 (23.2%)	5 (8.9%)	8 (34.8%)	.031	.5	.008
Tumor necrosis factor-α, pg/mL	41.6 (26.7–80.9)	33.1 (12.3–96.5)	44.7 (35.6–100.4)	.2	.3	.07
Macrophage migration inhibitory factor, ng/mL	2.4 (1.4–5.7)	2.7 (1.3–5.3)	2.2 (.9–14.6)	.9	>.9	.9
Interleukin-4 detectable*	27 (48.2%)	27 (48.2%)	14 (60.9%)	>.9	.3	.3
Interleukin-10, pg/mL	1738 (439–5258)	338 (140–1001)	3553 (1361–6,040)	<.0005**	.2	.0001**
b. Additional markers						
Interleukin-1α, pg/mL	86.4 (0.7–190.0)	5.7 (0.7–46.6)	107 (31–215)	<.0005**	.7	.0008**
Interleukin-1β detectable*	0 (0%)	1 (1.8%)	0 (0%)	.3	>.9	>.9
Interleukin-2 detectable*	6 (10.7%)	3 (5.4%)	0 (0%)	.3	.1	.6
Interleukin-5 detectable*	4 (7.1%)	3 (5.4%)	3 (13.0%)	.7	.4	.4
Interleukin-6, pg/mL	187 (76–438)	102 (24–274)	266 (171–834)	.013	.063	.0007**
Interleukin-7 detectable*	6 (10.7%)	7 (12.5%)	4 (17.4%)	.8	.4	.7
Interleukin-8, pg/mL	80.5 (31.3–250.0)	77.5 (19.2–393.8)	161 (36–371)	.8	.5	.6
Interleukin-13 detectable	2 (3.6%)	4 (7.1%)	1 (4.4%)	.4	.8	.6
Interelukin-15 detectable*	0 (0%)	1 (1.7%)	2 (7.1%)	.3	.026	.2
Interleukin-17 detectable*	4 (7.0%)	3 (5.1%)	0 (0%)	.7	.2	.6
Granulocyte colony-stimulating factor, pg/mL	86.9 (29.5–197.6)	65.5 (6.6–118.5)	118 (53–346)	.067	.2	.019
Granulocyte monocyte colony-stimulating factor detectable*	8 (14.3%)	10 (17.9%)	6 (26.1%)	.6	.2	.5
Eotaxin detectable*	0 (0%)	2 (3.4%)	0 (0%)	.2	>.9	>.9
Interferon inducible protein-10, pg/mL	994 (425–2242)	368 (209–549)	801 (380–2445)	<.0005**	.8	.001**
Monocyte chemoattractant protein-1, pg/mL	221 (107–534)	113 (61–237)	233 (132–393)	.004	.9	.009
Macrophage inflammatory protein-1α detectable*	3 (5.4%)	5 (8.9%)	0 (0%)	.4	.3	.3
sFas, pg/mL	2659 (1925–3975)	4321 (3532–5126)	2758 (2294–3773)	<.0005**	.5	.0005**
sFas ligand, pg/mL	342 (249–438)	337 (230–471)	402 (227–627)	.9	.4	.5
Tissue PAI-1, ng/mL	37 (28–50)	88 (42–147)	163 (58–254)	<.0005**	<.0005**	.14

NOTE. Results are given as median (interquartile range), unless otherwise indicated.

*Proportion with detectable level in number (%).

** The *P* value remains significant after adjustment for multiple comparisons.

concentration differed significantly in bivariate analysis, being markedly higher in children with cerebral malaria (Table 2b).

Pathway Analysis of Cerebral Malaria. In a pathway analysis of the clinical data and originally chosen immune markers lower platelet counts (standard $\beta = .40$; $P < .0001$), history of treatment with traditional herbal medicine (standard $\beta = .34$; $P < .0001$), and heme positive urine (standard $\beta = .25$; $P = .006$) were associated independently with cerebral malaria. The model had a good fit to data ($\chi^2 = .7$; $df = 3$; $P = .9$), and it was statistically significant (RMSEA = .0; 90% CI, .0–.05).

In an analysis that included both the original and additional immune markers, only tissue PAI-1 levels proved to make a significant and consistent addition to the model, with higher levels being associated with cerebral malaria (standard $\beta = .36$; $P < .0001$). In these models, cerebral malaria was a risk factor for seizures rather than seizures leading to cerebral malaria (standard $\beta = .61$; $P < .0001$).

These findings in pathway analysis were confirmed in a logistic regression model. In this model, the presence of hemoglobinuria was associated with an estimated 57.7-fold increase in the odds of cerebral malaria ($P = .005$), a history of traditional herbal medicine use with a 20.0-fold increase in the odds ($P = .007$), a decrease in the platelet count by 1 SD with a 4.0-fold increase in the odds ($P = .019$), and an increase in the tissue PAI-1 concentration by 1 SD with a 2.2-fold increase in the odds ($P = .050$).

Table 3. Comparison of Standard β (*P* Value) for Severe Anemia and for Reticulocyte Count from Pathway Analysis Models

	Severe Anemia	Reticulocyte Count
a. Clinical data and original markers		
Interleukin-10	-.66 (<.001)	.49 (<.001)
Tumor necrosis factor-α	.26 (.003)	-.32 (<.001)
Weight-for-age *z* score	-.18 (.008)	.16 (.030)
Spleen size	.17 (.006)	-.02 (.7)
Presence of stool parasites	.16 (.021)	-.20 (.006)
b. Clinical data and expanded markers		
Interleukin-10	-.61 (<.001)	.45 (<.001)
Tumor necrosis factor-α	.26 (.004)	-.31 (<.001)
SFas	.22 (.001)	-.12 (.10)
Weight-for-age *z* score	-.18 (.008)	.15 (.045)
Spleen size	.16 (.008)	-.02 (.7)
Presence of stool parasite	.15 (.031)	-.20 (.008)

DISCUSSION

The results of this study point to distinct pathways for severe malarial anemia and cerebral malaria among children from the Tonga ethnic group of southern Zambia, and they suggest that suppression of erythropoiesis is a principal mechanism that distinguishes severe malarial anemia from cerebral malaria. In particular, direct associations with severe malarial anemia were found for lower circulating IL-10 concentrations as well as for higher TNF-α and sFas concentrations, lower weight-for-age *z* scores, the presence of stool parasites, and splenomegaly. In contrast, factors directly associated with cerebral malaria included thrombocytopenia and elevated tissue PAI-1 concentrations as well as exposure to traditional herbal medicine and hemoglobinuria, a marker of intravascular hemolysis. Although many studies of severe malaria have pooled severe malarial anemia and cerebral malaria together [28–30], our findings support investigations emphasizing distinct immunologic and clinical features of these complications [31].

Severe Malarial Anemia

Mean corpuscular volumes, white blood cell counts, and platelet counts were not lower with severe anemia than with uncomplicated malaria, suggesting that iron-deficient erythropoiesis, megaloblastic changes, and generalized bone marrow suppression were unlikely to be major factors. These findings are therefore consistent with a recent study indicating that neither iron deficiency nor folic acid deficiency were prominent contributors to severe anemia in preschool children in Malawi [32]. Urinary detection of heme was not significantly greater with severe anemia in this study either, indicating that intravascular hemolysis was not a dominant contributing factor. Although HIV testing was not routinely performed in this study, the higher rather than lower total lymphocyte counts suggests that HIV infection was not a major cause of severe anemia.

Of 6 originally chosen immunologic markers, only lower plasma concentrations of IL-10 and higher concentrations of TNF-α were associated with severe anemia in pathway analysis. Consistent with some [27] but not all [33] investigations in other settings, lower nutritional status as reflected in the weight-for-age *z* score and therapy with sulfadoxine/pyrimethamine were associated with lower IL-10 concentrations in this analysis. Contrary to our initial hypotheses, significant associations with increased circulating levels of T_H1 cytokines or macrophage migration inhibitory factor were not observed. An inverse correlation of IL-10 with severe anemia was particularly strong. These findings from southern Zambia are consistent with clinical studies demonstrating an association of a low ratio of plasma IL-10 to TNF-α with severe malarial anemia in young children in Ghana and Kenya [6, 34] and the possibility that low IL-10 activity and increased TNF-α activity are associated with suppression of erythropoiesis in the setting of malaria [35]. Furthermore, recombinant TNF-α can mediate erythropoietic suppression [36] and IL-10 knockout mice infected with *Plasmodium chabaudi* displayed more anemia [37], which was reversed following TNF-α neutralization [38]. However, other studies have found no association between low interelukin 10 or high TNF-α and malarial anemia [39]. Of 19 additional immune markers tested, only sFas was associated with severe malarial anemia in pathway analysis, in addition to IL-10 and TNF-α. The apoptotic marker sFas is expressed in developing but not mature red blood cells [40], and increased apoptosis of erythroid precursors is a potential mechanism for decreased effective erythropoiesis in malaria.

Cerebral Malaria

Compared with uncomplicated malaria, cerebral malaria had the strongest associations in pathway analysis with lower platelet counts and elevated tissue PAI-1 concentrations, followed by exposure to traditional herbal medicine and heme-positive

urine. The combination of thrombocytopenia, elevated PAI-1 levels, intravascular hemolysis, and reversibly altered consciousness that characterizes cerebral malaria in this study has parallels to the noninfectious hematologic disorder thrombotic thrombocytopenic purpura [41, 42].

PAI-1 is the primary inhibitor in plasma of activators of plasminogen. Studies in mice and humans indicated that PAI-1 may be part of the body's response to control malaria [23, 43]. The histopathology of cerebral malaria includes cerebral microvascular thrombi or fibrin deposition in some studies [9–11] but not in others [44]. In addition to inhibition of fibrinolysis, PAI-1 influences the adhesion and migration of cells, angiogenesis, and the activation of transforming growth factor-β, and elevated levels may be associated with vascular endothelial dysfunction [45, 46].

Intravascular hemolysis may contribute to a hemolytic vasculopathy and cerebrovascular complications in noninfectious hemolytic anemias such as sickle cell disease, in part through scavenging of nitric oxide, a key modulator of microvascular function [47]. Our present findings add to a growing body of evidence that this process applies to cerebral malaria as well. Other investigators reported that children with cerebral malaria have low plasma levels of nitric oxide metabolites and L-arginine, a metabolic precursor of nitric oxide [48, 49], and impaired mononuclear cell NO synthase type 2 expression [49]. Furthermore, adults with severe malaria, predominantly manifested by coma, had vascular endothelial dysfunction that correlated with the degree of hemolysis and decreased nitric oxide bioavailability, and that was reversed by infusion of the precursor of nitric oxide, L-arginine [12]. Nitric oxide deficiency, in part because of increased nitric oxide scavenging by free plasma hemoglobin, may contribute to experimental cerebral malaria in mice [50].

CONCLUSIONS

Our study has a number of limitations. It was conducted under remote conditions in which not all desirable hematological and imaging studies could be performed. Circulating concentrations of cytokines may not reflect the degree to which bone marrow precursors and central nervous system cells are exposed to the influence of these mediators. Asymptomatic parasitemia is common, and in some patients the anemia or coma may have not been because of malaria per se, despite the presence of a positive malaria smear. Myoglobinuria in addition to hemoglobiniuria has been described in acute malaria, and our dipstick method could not distinguish between these 2 conditions. Data on other measures of hemolysis such as serum lactate dehydrogenase concentrations or schistocytes in the peripheral blood smear are not available. Further investigations of the observed associations of stool parasites with severe anemia and traditional herbal medicine with cerebral malaria also were not performed.

Despite these and other limitations, our findings are consistent with a view that the complications of severe malarial anemia and cerebral malaria are pathogenically distinct. Severe malarial anemia is a disorder of selective suppression of erythroid progenitors in the setting of accelerated destruction of infected red blood cells. Contributing factors may include (1) inadequate production of IL-10 to protect erythroid precursors from the suppressive effects of proinflammatory cytokines, (2) suppression of erythroid progenitor cells by increased activity of TNF-α, and (3) underlying malnutrition and helminthic infestation. In contrast, cerebral malaria may be characterized by intravascular hemolysis and thrombocytopenia. In practical terms, our findings suggest that malaria control programs should expand efforts to correct underlying poor nutrition and helminthic infestations and to improve availability of effective antimalarial treatment. Furthermore, continued research is needed to better understand the complex immune response and vascular abnormalities related to human *P. falciparum* infection. In addition, efforts to prevent or treat severe malarial anemia by immune manipulation, and cerebral malaria by protection of the vascular endothelium, should be subjects for future research.

Funding

This work was supported by the National Institute of Allergy and Infectious Diseases at the National Institutes of Health (grants 1 R01 AI44857 and AI051306); the National Heart, Lung, and Blood Institute and the Office of Research on Minority Health at the National Institutes of Health (grant UH1-HL03679); and the National Institute of Research Resources, Howard University General Clinical Research Center (grant MO1-RR10284).

Acknowledgments

Philip E. Thuma participated in designing and performing the research and writing the paper. Janneke van Dijk participated in performing the research and writing the paper. Rick Bucala participated in designing the research and writing the paper. Zufan Debebe participated in performing the research and writing the paper. Sergei Nekhai participated in performing the research and writing the paper. Thea Kuddo participated in designing and performing the research and writing the paper. Mehdi Nouraie participated in analyzing the data and writing the paper. Günter Weiss participated in designing and performing the research and writing the paper. Victor R. Gordeuk participated in designing and performing the research, analyzing the data, and writing the paper.

References

1. McDevitt MA, Xie J, Gordeuk V, Bucala R. The anemia of malaria infection: Role of inflammatory cytokines. Curr Hematol Rep **2004**; 3:97–106.
2. Wickramasinghe SN, Abdalla SH. Blood bone marrow changes in malaria. Baillieres Best Pract Res Clin Haematol **2000**; 13:277–99.
3. Biemba G, Gordeuk VR, Thuma PE, Mabeza GF, Weiss G. Prolonged macrophage activation and persistent anaemia in children with complicated malaria. Trop Med Int Health **1998**; 3:60–5.
4. Nussenblatt V, Mukasa G, Metzger A, Ndeezi G, Garrett E, Semba RD. Anemia and interleukin-10, tumor necrosis factor α, and erythropoietin levels among children with acute, uncomplicated Plasmodium

falciparum malaria. Clin Diagn Lab Immunol **2001**; 8: 1164–70.
5. McDevitt MA, Xie J, Shanmugasundaram G, et al. A critical role for the host mediator macrophage migration inhibitory factor in the pathogenesis of malarial anemia. J Exp Med **2006**; 203:1185–96.
6. Kurtzhals JA, Adabayeri V, Goka BQ, et al. Low plasma concentrations of interleukin 10 in severe malarial anaemia compared with cerebral uncomplicated malaria. Lancet **1998**; 351:1768–72.
7. Lyke KE, Burges R, Cissoko Y, et al. Serum levels of the proinflammatory cytokines interleukin-1 β (IL-1β), IL-6, IL-8, IL-10, tumor necrosis factor α, IL-12(p70) in Malian children with severe *Plasmodium falciparum* malaria and matched uncomplicated malaria or healthy controls. Infect Immun **2004**; 72:5630–7.
8. Grau GE, Piguet PF, Vassalli P, Lambert PH. Tumor-necrosis factor and other cytokines in cerebral malaria: Experimental and clinical data. Immunol Rev **1989**; 112:49–70.
9. Krishnan A, Karnad DR, Limaye U, Siddharth W. Cerebral venous and dural sinus thrombosis in severe falciparum malaria. J Infect **2004**; 48:86–90.
10. MacPherson GG, Warrell MJ, White NJ, Looareesuwan S, Warrell DA. Human cerebral malaria: A quantitative ultrastructural analysis of parasitized erythrocyte sequestration. Am J Pathol **1985**; 119: 385–401.
11. Boonpucknavig V, Boonpucknavig S, Udomsangpetch R, Nitiyanant P. An immunofluorescence study of cerebral malaria: A correlation with histopathology. Arch Pathol Lab Med **1990**; 114:1028–34.
12. Yeo TW, Lampah DA, Gitawati R, et al. Impaired nitric oxide bioavailability L-arginine reversible endothelial dysfunction in adults with falciparum malaria. J Exp Med **2007**; 204:2693–704.
13. Molyneux ME, Taylor TE, Wirima JJ, Borgstein A. Clinical features and prognostic indicators in paediatric cerebral malaria: A study of 131 comatose Malawian children. Q J Med **1989**; 71:441–59.
14. Chaisavaneeyakorn S, Othoro C, Shi YP, et al. Relationship between plasma Interleukin-12 (IL-12) IL-18 levels and severe malarial anemia in an area of holoendemicity in western Kenya. Clin Diagn Lab Immunol **2003**; 10:362–6.
15. Grau GE, Heremans H, Piguet PF, et al. Monoclonal antibody against interferon gamma can prevent experimental cerebral malaria its associated overproduction of tumor necrosis factor. Proc Natl Acad Sci U S A **1989**; 86:5572–4.
16. Ouma C, Davenport GC, Awandare GA, et al. Polymorphic variability in the interleukin (IL)-1β promoter conditions susceptibility to severe malarial anemia functional changes in IL-1β production. J Infect Dis **2008**; 198:1219–26.
17. Ramharter M, Kremsner PG, Willheim M, Winkler H, Graninger W, Winkler S. Plasmodium falciparum-specific interleukin-2 and tumor necrosis factor-α expressing-T cells are associated with resistance to reinfection and severe malaria in healthy African children. Eur Cytokine Netw **2004**; 15:189–96.
18. Ohashi J, Naka I, Patarapotikul J, Hananantachai H, Looareesuwan S, Tokunaga K. A single-nucleotide substitution from C to T at position -1055 in the IL-13 promoter is associated with protection from severe malaria in Thailand. Genes Immun **2003**; 4:528–31.
19. Gosi P, Khusmith S, Looareesuwan S, et al. Complicated malaria is associated with differential elevations in serum levels of interleukins 10, 12, 15. Southeast Asian J Trop Med Public Health **1999**; 30:412–7.
20. Griffith JW, O'Connor C, Bernard K, Town T, Goldstein DR, Bucala R. Toll-like receptor modulation of murine cerebral malaria is dependent on the genetic background of the host. J Infect Dis **2007**; 196:1553–64.
21. Stoiser B, Looareesuwan S, Thalhammer F, et al. Serum concentrations of granulocyte-colony stimulating factor in complicated *Plasmodium falciparum* malaria. Eur Cytokine Netw **2000**; 11:75–80.
22. Grau GE, Kindler V, Piguet PF, Lambert PH, Vassalli P. Prevention of experimental cerebral malaria by anticytokine antibodies. Interleukin 3 and granulocyte macrophage colony-stimulating factor are intermediates in increased tumor necrosis factor production and macrophage accumulation. J Exp Med **1988**; 168:1499–504.
23. Mohanty D, Ghosh K, Nandwani SK, et al. Fibrinolysis, inhibitors of blood coagulation, monocyte-derived coagulant activity in acute malaria. Am J Hematol **1997**; 54:23–9.
24. Aiello FB, Keller JR, Klarmann KD, Dranoff G, Mazzucchelli R, Durum SK. IL-7 induces myelopoiesis and erythropoiesis. J Immunol **2007**; 178:1553–63.
25. Camacho LH, Wilairatana P, Weiss G, et al. The eosinophilic response haematological recovery after treatment for *Plasmodium falciparum* malaria. Trop Med Int Health **1999**; 4:471–5.
26. Mulenga M, VangGeertruyden JP, Mwananyanda L, et al. Safety efficacy of lumefantrine-artemether (Coartem) for the treatment of uncomplicated *Plasmodium falciparum* malaria in Zambian adults. Malar J **2006**; 5:73.
27. Van der Werff Ten Bosch J, Schotte P, Ferster A, et al. Reversion of autoimmune lymphoproliferative syndrome with an antimalarial drug: Preliminary results of a clinical cohort study molecular observations. Br J Haematol **2002**; 117:176–88.
28. Okech B, Mujuzi G, Ogwal A, Shirai H, Horii T, Egwang TG. High titers of IgG antibodies against *Plasmodium falciparum* serine repeat antigen 5 (SERA5) are associated with protection against severe malaria in Ugandan children. Am J Trop Med Hyg **2006**; 74:191–7.
29. Clark TG, Diakite M, Auburn S, et al. Tumor necrosis factor lymphotoxin-α polymorphisms and severe malaria in African populations. J Infect Dis **2009**; 199:569–75.
30. Yacoub S, Lang HJ, Shebbe M, et al. Cardiac function and hemodynamics in Kenyan children with severe malaria. Crit Care Med **2010**; 38:940–5.
31. Dobano C, Rogerson SJ, Mackinnon MJ, et al. Differential antibody responses to *Plasmodium falciparum* merozoite proteins in Malawian children with severe malaria. J Infect Dis **2008**; 197:766–74.
32. Calis JC, Phiri KS, Faragher EB, et al. Severe anemia in Malawian children. N Engl J Med **2008**; 358:888–99.
33. Hillyer L, Dao B, Niemiec P, et al. Elevated bioactivity of the tolerogenic cytokines, interleukin-10 transforming growth factor-β, in the blood of acutely malnourished weanling mice. Exp Biol Med (Maywood) **2006**; 231:1439–47.
34. Othoro C, Lal AA, Nahlen B, Koech D, Orago AS, Udhayakumar V. A low interleukin-10 tumor necrosis factor-α ratio is associated with malaria anemia in children residing in a holoendemic malaria region in western Kenya. J Infect Dis **1999**; 179:279–82.
35. Robson KJ, Weatherall DJ. Malarial anemia STAT6. Haematologica **2009**; 94:157–9.
36. Johnson RA, Waddelow TA, Caro J, Oliff A, Roodman GD. Chronic exposure to tumor necrosis factor in vivo preferentially inhibits erythropoiesis in nude mice. Blood **1989**; 74:130–8.
37. Linke A, Kühn R, Müller W, Honarvar N, Li C, Langhorne J. *Plasmodium chabaudi chabaudi*: Differential susceptibility of gene-targeted mice deficient in IL-10 to an erythrocytic-stage infection. Exp Parasitol **1996**; 84:253–63.
38. Li C, Sanni LA, Omer F, Riley E, Langhorne J. Pathology of *Plasmodium chabaudi chabaudi* infection and mortality in interleukin-10-deficient mice are ameliorated by anti-tumor necrosis factor α and exacerbated by anti-transforming growth factor β antibodies. Infect Immun **2003**; 71:4850–6.
39. Helleberg M, Goka BQ, Akanmori BD, Obeng-Adjei G, Rodriques O, Kurtzhals JA. Bone marrow suppression and severe anaemia associated with persistent *Plasmodium falciparum* infection in African children with microscopically undetectable parasitaemia. Malar J **2005**; 4:56.
40. Lamikanra AA, Brown D, Potocnik A, Casals-Pascual C, Langhorne J, Roberts DJ. Malarial anemia: Of mice and men. Blood **2007**; 110:18–28.
41. Chinowsky MS. Cerebral falciparum malaria mimicking thrombotic thrombocytopenic purpura. South Med J **1991**; 84:374–8.
42. Anthony MT, Zeigler ZR, Lister J, et al. Plasminogen activator inhibitor (PAI-1) antigen levels in primary TTP secondary TTP post-bone marrow transplantation. Am J Hematol **1998**; 59:9–14.

43. Krücken J, Dkhil MA, Braun JV, et al. Testosterone suppresses protective responses of the liver to blood-stage malaria. Infect Immun **2005**; 73:436–43.
44. Pongponratn E, Riganti M, Harinasuta T, Bunnag D. Electron microscopy of the human brain in cerebral malaria. Southeast Asian J Trop Med Public Health **1985**; 16:219–27.
45. Irigoyen JP, Muñoz-Cánoves P, Montero L, Koziczak M, Nagamine Y. The plasminogen activator system: Biology and regulation. Cell Mol Life Sci **1999**; 56:104–32.
46. Fay WP, Garg N, Sunkar M. Vascular functions of the plasminogen activation system. Arterioscler Thromb Vasc Biol **2007**; 27:1231–7.
47. Kato GJ, Hsieh M, Machado R, et al. Cerebrovascular disease associated with sickle cell pulmonary hypertension. Am J Hematol **2006**; 81:503–10.
48. Lopansri BK, Anstey NM, Weinberg JB, et al. Low plasma arginine concentrations in children with cerebral malaria decreased nitric oxide production. Lancet **2003**; 361:676–8.
49. Anstey NM, Weinberg JB, Hassanali MY, et al. Nitric oxide in Tanzanian children with malaria: Inverse relationship between malaria severity nitric oxide production/nitric oxide synthase type 2 expression. J Exp Med **1996**; 184:557–67.
50. Gramaglia I, Sobolewski P, Meays D, et al. Low nitric oxide bioavailability contributes to the genesis of experimental cerebral malaria. Nat Med **2006**; 12:1417–22.