

ASSOCIATION OF INTRALEUKOCYTIC *PLASMODIUM FALCIPARUM* MALARIA PIGMENT WITH DISEASE SEVERITY, CLINICAL MANIFESTATIONS, AND PROGNOSIS IN SEVERE MALARIA

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Abstract. Peripheral parasite density of *Plasmodium falciparum* is used as an indicator of malaria disease severity, but does not quantify central sequestration, which is important in the pathogenesis of severe disease. Malaria pigment, recognizable within the cytoplasm of phagocytic cells by light microscopy may represent a peripheral marker for parasite biomass. One hundred seventy-two index cases of severe malaria and 172 healthy age-, residence-, and ethnicity-matched controls with uncomplicated malaria in Bandiagara, Mali were analyzed prospectively for presence of malaria pigment. The presence of polymorphonuclear cell (PMN) and monocyte pigment was strongly associated with severe disease compared with uncomplicated malaria. Total PMN pigment burden in children with severe malaria was higher in those with cerebral manifestations and with combined cerebral manifestations and severe anemia (hemoglobin ≤ 5 g/dL) but was not associated with hyperparasitemia ($> 500,000$ asexual forms/mm³). Additionally, pigmented PMNs/mm³ was associated with a fatal outcome in patients with severe malaria. This study validates the presence of malaria pigment in monocytes and neutrophils as a marker for disease severity, and demonstrates that pigmented neutrophils are associated with cerebral malaria and with death in children with severe malaria.

INTRODUCTION

The pathophysiology of severe *Plasmodium falciparum* malaria is complex and results in a broad spectrum of disease manifestations.^{1,2} Sequestration of parasites is thought to be central to these disease processes, but anemia, cytokine production, and metabolic distortion may also contribute to the spectrum of disease.^{3–5} Parasite density generally correlates with disease severity, but peripheral parasitemia does not necessarily reflect the burden of sequestered parasites. In hospital-based studies, malaria therapy taken prior to admission may further hamper efforts to properly diagnose and characterize acute illness. Methods of estimating malaria disease severity and predicting prognosis may be useful in stratifying patients in the early stages of admission and assessment.

Hemozoin, also known as malaria pigment, is a product of hemoglobin digestion by *Plasmodia*.⁶ The existence of hemozoin has been recognized for centuries, but the complexities of its formation and its biologic and clinical relevance are incompletely understood. As part of parasite erythrocytic invasion, hemoglobin is proteolyzed releasing toxic heme. Due to the absence of heme oxygenase, *Plasmodia* are unable to cleave heme into open-chain tetrapyrrole to allow for cellular excretion.⁷ To detoxify soluble heme, a novel breakdown product known as hemozoin is created intracellularly. This digestive end-product of hemoglobin is sequestered in the *P. falciparum* digestive vacuole within infected red blood cells and released into host circulation during schizogony. Hemozoin is composed of Fe^{III}-porphyrin units linked by propionate oxygen-iron bonds into polymers accompanied by additional host and parasite nucleic acids and lipids.^{8,9} Once this insoluble polymer is released into host circulation, scavenger neutrophils and monocytes phagocytose the material. It is easily visible by light microscopy, appearing as a black, brown, or amber pigment or as a birefringent crystal under polarized light^{10,11} (Figures 1 and 2). Given that the typical half-life of a neutrophil is 6–8 hours and that of a monocyte

is several days, the quantity and distribution of engulfed pigment within these phagocytic cells may reflect the chronology of a patient's infection.

In the past decade, investigators have demonstrated the utility of assessing pigment formation for disease characterization. The presence of pigment correlates with mortality of severe malaria in Asian adults from an area hypoendemic for disease¹² and with disease severity in African children.^{13–15} We sought to expand and validate these clinical findings through a matched case-control study in Malian children in which we assessed the presence and quantity of malaria pigment and its association with disease severity, clinical manifestations and prognosis.

METHODS AND MATERIALS

Study site and enrollment. Blood films were obtained from 516 Malian children (age range = 3 months to 14 years) on enrollment into a case-control study evaluating risk and protective factors for severe malaria. The study site of Bandiagara is located in east central Mali and has intense seasonal transmission (July–December) of *P. falciparum* malaria. The dominant ethnic group is Dogon (80%) with Peuhl (10%), Bambara (3%), and other ethnic groups (7%) also present. One hundred seventy-two index cases of severe malaria from Bandiagara and surrounding areas were admitted to the Bandiagara Malaria Project ward from July 2000 to December 2001. Cases were classified as severe malaria based on modified criteria put forth by the World Health Organization¹ (Table 1). More than one clinical diagnosis for severe malaria was possible, but cerebral malaria and severe anemia were considered to be the primary defining features when they co-existed with other criteria. Each index case was age-, residence-, and ethnicity-matched to a case of uncomplicated malaria and a healthy control. Age categories were defined as 3–5 months, 6–11 months, 1 year, 2 years, 3–4 years, 5–6

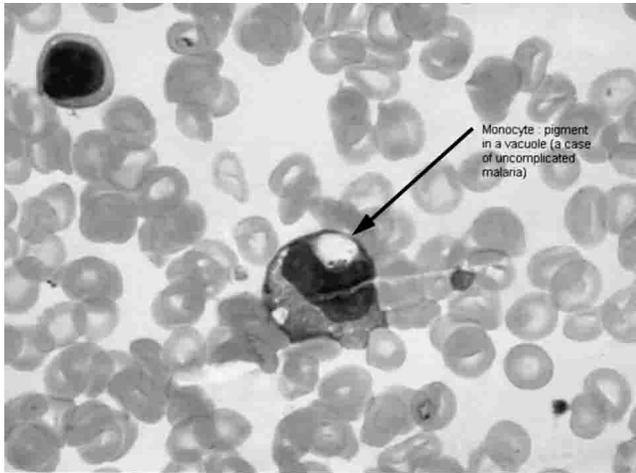


FIGURE 1. Birefringent amber crystals (**arrow**) visualized by light microscopy within a monocytic vacuole in a case of uncomplicated malaria from Mali.

years, 7–8 years, 9–10 years, 11–12 years, and 13–14 years. Residence was defined as one of eight distinct sectors of Bandiagara town or, in the case of children from outer villages, the specific village of origin. Uncomplicated malaria was defined as *P. falciparum* parasitemia and an axillary temperature $\geq 37.5^{\circ}\text{C}$ detected by active surveillance, or parasitemia and symptoms leading to treatment-seeking behavior in the absence of other clear cause of fever on passive surveillance. Matched uncomplicated malaria controls were enrolled from the population of children presenting to the daily Bandiagara Malaria Project clinic. Healthy controls were enrolled after traveling to the home of the child with severe malaria and following a standardized routine of exiting the front entrance of a compound and making consecutive left turns until another compound with an eligible control was identified. Children were enrolled as healthy controls if they were asymptomatic for acute illness, had no evidence or history of chronic illness, and if they were found to be a parasitemic upon examination. The study protocol was reviewed and approved by

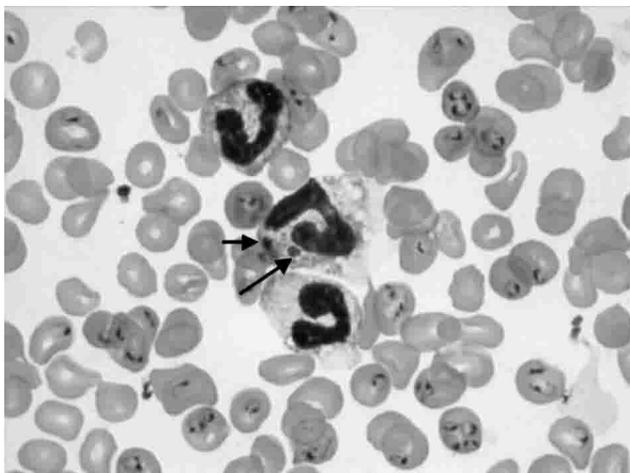


FIGURE 2. Amber intracytoplasmic hemozoin (**arrows**) visualized by light microscopy within a polymorphonuclear cell in a case of severe malaria with hyperparasitemia from Mali.

the University of Mali and the University of Maryland Institutional Review Boards. Written informed consent was obtained from parents or guardians of all study participants.

Assessment of pigment. Thick and thin blood films obtained at enrollment prior to therapy were stained with Giemsa. Peripheral parasite density was determined from thick films based on the number of asexual forms/ mm^3 per 300 leukocytes and an assumed white blood cell (WBC) count of $7,500 \text{ cells}/\text{mm}^3$. Parasite density calculations were performed immediately and results were used for severe disease stratification. Thin smears were analyzed in groups with absolute WBC and differential counts determined manually by two microscopists. Acceptable degrees of variation have been noted between automated and manual WBC counts and differential counts with a high correlation between the two when analyzed using a Coulter (Hiialeah, FL) JR apparatus.^{16,17} Malaria pigment was detected on thin films by counting 100 polymorphonuclear cells (PMNs) and 30 monocytes and determining the quantity of cells containing pigment. A ratio was then determined of total pigmented PMNs/100 or monocytes/30. Microscopists were blinded to clinical presentation and outcome. To determine interobserver variability, 10% of the slides were re-examined by a second microscopist. To standardize total pigment burden across variable absolute WBCs and differential counts as determined from thin smears, total PMN and monocyte pigment per mm^3 were calculated as follows: Total pigmented PMN/ $\text{mm}^3 = (\text{number of pigmented PMNs}/100) \times (\text{absolute WBCs} \times \text{percent of PMNs})$. Similarly, total pigmented monocytes/ $\text{mm}^3 = (\text{number of pigmented monocytes}/30) \times (\text{absolute WBCs} \times \text{percent of monocytes})$. When the percent of monocytes in the differential cell count was less than 1% but pigment was noted on monocyte pigment count, an arbitrary monocyte differential count of 0.5% ($n = 9$) was assigned.

Statistical analysis. Pooled analyses between clinical groups were made using a two-sided Student's *t*-test for continuous variables with equal variance and a chi-square test for categorical variables using Stata version 7.0 (Stat Corp., College Station, TX) and SPSS version 10.0 (SPSS, Inc., Chicago, IL). For the purposes of analyzing differences between matched pairs, McNemar's test was used to assess absolute pigment counts and pigment/ mm^3 using Stata version 7.0. A

TABLE 1

Defining features of severe malaria as published by the World Health Organization

Coma (Blantyre Coma Scale [BCS] ≤ 2)
Seizure (one or more witnessed by the investigators)
Obtundation (depressed consciousness with BCS > 2)
Parasitemia $\geq 500,000/\text{mm}^3$
Lethargy or prostration (clinical judgment or child ≥ 7 months old unable to sit unassisted)
Severe anemia (hemoglobin $\leq 5 \text{ g/dL}$)
Respiratory distress (intercostal muscle retraction, deep breathing, grunting)
Hypoglycemia (glucose $\leq 40 \text{ mg/dL}$)
Jaundice
Renal insufficiency as indicated by lack of urination for ≥ 1 day
Gross hematuria
Inability to eat or drink
State of shock (systolic blood pressure $\leq 50 \text{ mm of Hg}$, rapid pulse, cold extremities)
Repeated vomiting

level of statistical significance (two-sided) was set at $P < 0.05$. A Kappa coefficient was used in assessing inter-observer variability in the reading of a 10% proportion of thin smears.

RESULTS

Clinical characteristics. One hundred seventy-two cases each of severe malaria, uncomplicated malaria, and healthy controls were enrolled in the study and evaluated. The patient characteristics are listed in Table 2. Fifteen of 172 patients with severe malaria (8.7%) died. Of those with severe malaria, 49% were hyperparasitemic, 45% had cerebral malaria, 16% were severely anemic, and 2.4% met other criteria. Patients meeting the criteria for both cerebral malaria and severe anemia ($n = 15$) were analyzed as a unique subset. Anemia was more common among those with a fatal outcome than in survivors (mean hemoglobin concentration = 5.9 g/dL versus 8.2 g/dL, respectively; $P < 0.0001$). Additionally, geometric mean parasite densities in children with severe malaria were significantly lower in those that died than in survivors (29,994 asexual forms/mm³ versus 174,428 asexual forms/mm³; $P < 0.0001$). When patients whose sole criterion for severe malaria was hyperparasitemia (mortality = 0.01%) were excluded, the geometric mean parasite density in survivors was 69,278 asexual forms/mm³ compared with 29,994 asexual forms/mm³ in those who died ($P = 0.06$). No significant differences were noted among study groups in age distribution, sex, or total WBC count.

Malaria pigment measurements. Of 172 cases enrolled in each group, thin films were available for 163 of the severe malaria cases, 163 of the uncomplicated malaria controls, and 164 of the healthy controls. Ten percent of the films were counted by a second observer and the interobserver variability was determined (kappa coefficient [κ] = 0.88 for PMN agreement, $\kappa = 0.77$ for monocytes). Total hemozoin counts are summarized in Table 3. The results are depicted graphically in a scatter plot in Figure 3.

The proportions of both neutrophils and monocytes with malaria pigment were higher in patients with severe malaria than with uncomplicated malaria (PMNs = 349 cells/mm³ versus 64 cells/mm³; $P < 0.0001$, monocytes = 219 cells/mm³ versus 94 cells/mm³; $P = 0.003$). Similarly, phagocytic cells from uncomplicated malaria cases had more pigment than those from healthy controls (PMNs = 64 cells/mm³ versus 1.1 cells/mm³; $P < 0.0001$, monocytes = 94 cells/mm³ versus 4.9 cells/mm³; $P < 0.0001$).

Using the matched design (age, residence, and ethnicity) of the study population, we performed McNemar's testing to assess differences between individually matched pairs. An increased amount of malaria pigment in phagocytic cells was more likely in severe malaria cases compared with uncomplicated malaria cases (pigmented PMNs: odds ratio [OR] = 5.6, 95% confidence interval [CI] = 2.83–12.31, $P < 0.0001$, pigmented monocytes: OR = 2.85, 95% CI = 1.38–5.85, $P = 0.003$). Within the severe malaria group, an average of 635 pigmented PMNs/mm³ were noted at admission in subjects who subsequently died due to severe disease, compared with an average of 320 pigmented PMNs/mm³ in the survivors ($P = 0.02$). A trend was noted, but did not achieve statistical significance, when the same analysis was performed evaluating pigment presence in monocytes in severe malaria survivors versus children who died of severe disease (407 cells/mm³ versus 198 cells/mm³; $P = 0.09$).

To evaluate association between presence of pigment and disease syndrome, the severe malaria group was stratified into the four predominant presenting diagnoses: cerebral malaria ($n = 77$), severe anemia ($n = 26$), severe anemia and cerebral malaria ($n = 15$), and hyperparasitemia ($n = 85$). For subjects in both the cerebral malaria group and the combined cerebral malaria/severe anemia group, significantly more pigmented PMNs/mm³ were noted at presentation than in patients with severe malaria without cerebral symptoms (Table 4). No such association with clinical syndrome was found for monocytic pigment. When stratified by parasitemia (those with peripheral parasite densities $\geq 500,000$ asexual forms/mm³ and those with $< 500,000$) no significant differences were noted either in amount of pigmented PMNs or monocytes/mm³. A higher concentration of monocyte pigment was noted in children with severe malaria who were not hyperparasitemic compared with those who were (290 cells/mm³ versus 143 cells/mm³; $P = 0.04$). Multivariate regression analysis found no significant association between pigmented monocytes/mm³ and peripheral parasite density ($P = 0.11$). Similarly, no association was noted between polymorphonuclear cell pigment and level of parasitemia ($P = 0.68$). A separate analysis was performed recalculating peripheral parasitemia based on the absolute WBC count determined on a thin smear rather than the standardized WBC count (7,500 cells/mm³ in this study) that is typically used in field trials. Peripheral parasite density results were not significantly different than those calculated using the standard WBC count, and no association was noted between PMN pigment and level of parasitemia.

TABLE 2

Patient characteristics at enrollment in age-, residence-, and ethnicity-matched severe malaria, uncomplicated malaria, and healthy control groups

Characteristics	Severe malaria		Uncomplicated malaria	Healthy control
	Survived	Died		
Number	157	15	172	172
Females (%)	74 (47)	8 (53)	85 (49.4)	76 (44.2)
Age in months (range)	39.4 (3–125)	27.5 (8–99)	39.4 (6–113)	37.8 (4–121)
Hemoglobin g/dL (range)	8.2 (2.6–12.9)	5.9 (2.6–7.4)†	9.3 (5.3–13.8)†	10.5 (6.2–14.5)†
WBC (mm ³)	14,093	16,178	17,560	13,887
Parasite density‡	174,428	29,927†	8,200†	0†

* Paired t-test were performed between healthy controls and uncomplicated malaria controls, between uncomplicated malaria and severe malaria patients, and between children that survived and those that died of severe disease. WBC = white blood cell.

† $P < 0.001$.

‡ Geometric mean (asexual forms/mm³).

TABLE 3

Hemozoin characteristics noted in age-, residence-, and ethnicity-matched cases of severe malaria, uncomplicated malaria, and aparasitemic healthy controls*

Characteristic	Severe malaria	Uncomplicated malaria	Healthy control
Number	163	163	164
Pigment-containing PMNs			
Patients with pigment (%)	140 (85.9)	88 (54)	3 (1.8)
Pigment/mm ³ (range)	349 (0–3,721)	64 (0–534)	1.1 (0–81)
Mean cells pigmented (%)	4.4	1.7	0.03
Pigment-containing monocytes			
Patients with pigment (%)	139 (85.2)	114 (69.9)	10 (6.1)
Pigment/mm ³ (range)	216 (0–3,420)	94 (0–1,698)	4.9 (0–285)
Mean cells pigmented (%)	14.4	5.4	0.03

* PMNs = polymorphonuclear phagocytic cells. Pigment/mm³ refers to the calculated amount of pigment-containing cells in 100 PMNs counted or in 30 monocyte cells counted on a blood thin smear multiplied by the absolute white blood cell count and the percentage of PMNs or monocytes determined on a differential cell count.

DISCUSSION

Peripheral *P. falciparum* parasite density alone, while necessary for the diagnosis of malarial infection, provides limited information about disease severity, manifestations, or outcomes. The distinction between malaria infection and disease is complex. Our understanding is limited by incomplete knowledge of how host factors including immunity, behavior, genetics, and susceptibility interact with parasite pathogenesis and genetic polymorphisms.² Diagnostic capabilities in the field are often limited to stained thick and thin blood smears. Results of these smears may be altered by partial or insufficient drug therapy. Methods of extracting additional information from these smears to aid in diagnosis or disease stratification could be useful in directing scarce treatment resources to those who need them most.

Clinical evidence supports a role for hemozoin as an indicator for disease severity in both children and adults and for prognosis in adults.^{12–15} Additionally, malaria pigment itself may possess physiologic properties that contribute to the course of disease. Natural pigment has been demonstrated *in vitro* to induce production of both tumor necrosis factor- α and interleukin-1- β ; this effect is ameliorated by protease digestion, suggesting the role of uncharacterized proteins.¹⁸ Monocytic cell dysfunction has been demonstrated (inhibition of oxidative burst, inability to digest hemozoin, or repeatedly phagocytose).^{19,20}

In the present study, we used a matched case-control design to explore hemozoin characteristics as it relates to severe malarial disease. Because of the broad range of peripheral WBC counts (1,700–44,500 cells/mm³), and the disparity between neutrophil and monocyte percentages on absolute differential assessment, we standardized hemozoin measurements among groups by assessing the total amount of malaria pigment/mm³. We first analyzed groups by quantifying the percentage of pigmented monocyte and polymorphonuclear cells. Consistent with previous studies, differences were noted between patients with severe and uncomplicated malaria and aparasitemic controls. By analyzing the data as a calculated amount of pigment/mm³, a significantly higher amount of pigment for both neutrophils and monocytes was again observed between the severe malaria group and the matched uncomplicated malaria controls, as well as between the uncomplicated malaria group and matched healthy controls. These results validate the correlation of malaria pigment and disease severity.

Malaria pigment has been found to correlate with death in adults, but this has not been substantiated in children. We analyzed the role of hemozoin as a prognostic indicator of severe malaria. After stratifying the severe malaria cases into children who died versus those who survived, a significantly greater amount of pigmented PMNs/mm³ was observed in the group who died. These findings mark the first time that neutrophilic pigment presence has proven a prognostic indicator for severe malaria mortality in children. No difference was detected in pigmented monocytes/mm³.

To illuminate differences between categories of severe malaria, subjects were stratified into one of the three predominant admission diagnoses; cerebral malaria, severe anemia and hyperparasitemia. A subset analysis was performed on children with the combined diagnosis of cerebral malaria with severe anemia. Children with cerebral malaria had more pigmented PMNs/mm³ on admission. Conversely, more pigmented monocytes were noted in children with severe anemia. Of the 26 children with severe anemia, 15 had concomitant cerebral malaria. As in children with cerebral malaria alone, those with both diagnoses had more pigmented PMNs/mm³ but no increase in pigmented monocyte/mm³.

In children with severe anemia as a sole diagnosis (n = 11), pigmented monocytes were significantly increased; this was also noted in children with severe anemia combined with other manifestations of severe malaria. It is possible that the low numbers of children with anemia as a sole diagnosis did not provide adequate power to detect an association between hemoglobin and pigmented PMNs/mm³. It is also possible that the pathophysiology of acute cerebral malaria and severe anemia may, in and of itself, have properties creating a milieu for increased pigment formation independent of either manifestation alone.

The reasons for variability in monocyte and neutrophil pigment in different disease manifestations of severe malaria remain unclear. The proportion of neutrophils and monocytes containing malaria pigment is affected by total parasite burden and synchronicity of the parasite life cycle, and the clearance kinetics of these pigmented cells may be inherently different. Cellular clearance kinetic studies performed by Day and others have shown peripheral pigment-containing neutrophil clearance times of 72 hours (range = 49–95 hours) and peripheral pigment-containing monocyte clearance of 216 hours (range = 180–240 hours). While clearance of pigmented monocytes appears to follow first-order kinetics, that

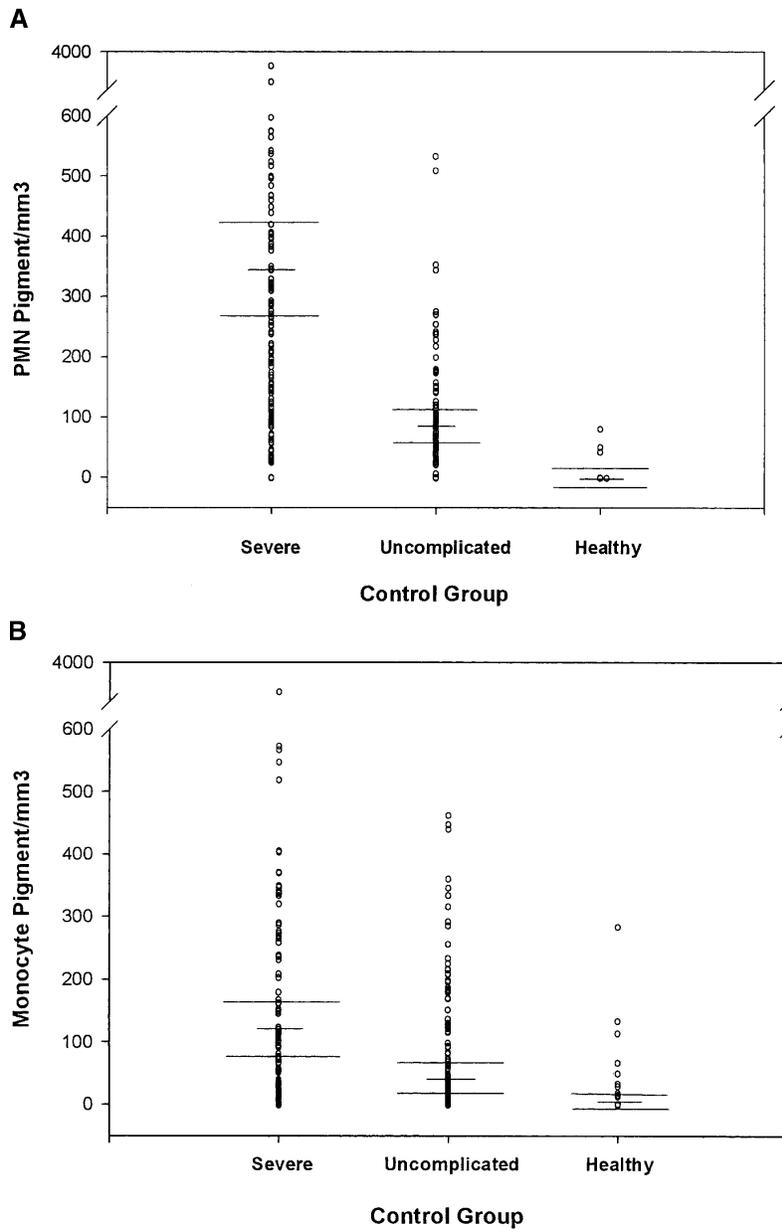


FIGURE 3. Scatterplot graph of **A**, polymorphonuclear cell (PMN) or **B**, monocyte pigment/ mm^3 distribution among study subjects with severe malaria, uncomplicated malaria, or no malaria. The standard error bars show 95% confidence intervals about the means, which are shown by the short cross-hatch bars.

of pigmented neutrophils departs from first-order kinetics, with increased rates of clearance at a lower cell density. The presence of pigmented neutrophils, with the rapid turnover of PMNs, may indicate a recent heavy parasitic burden and provide prognostic indications of disease, while longer-lived pigmented monocytes with longer clearance rates may reflect a more protracted infection or repeated infections.²¹

Malaria-induced anemia is multifactorial with hemolysis occurring more frequently in non-immune children and dyserythropoiesis occurring more often in regions with frequent and recurrent infections. The predominance of monocytic pigment in our anemic children may suggest that the profound anemia that occurred over a more protracted time period.²² The elevated pigmented polymorphonuclear cells in children with both cerebral malaria and severe anemia com-

pared with those with cerebral malaria alone may relate to the acuity of the illness with cerebral manifestations coupled with acute hemolysis.²¹ Thus, we hypothesize that pigmented monocytes indicate a protracted or indolent infection, while the factors leading to severe disease and death may be more fulminant and reflected in pigmented neutrophils. Longitudinal studies with serial pigment analysis would be better suited to address these hypotheses.

The use of hyperparasitemia as a criterion for severe malaria is recommended by the World Health Organization, but the clinical course in children who present with this manifestation appears less acute. At our study site, children whose sole defining criterion for severe malaria was hyperparasitemia appear to fall somewhere between uncomplicated malaria and severe malaria in the spectrum of illness. We noted

TABLE 4
Association of polymorphonuclear phagocytic cells (PMNs) and monocyte pigment with manifestations of severe disease in 163 patients*

Category	Patients	Cell	Mean pigmented cells/mm ³ (95% CI)	P
Cerebral vs. non-cerebral	77	PMN	448 (298–598)	0.018†
		Monocyte	260 (196–324)	0.80
Severe anemia vs. hemoglobin ≥ 5 g/dL	26	PMN	226 (99–318)	0.066
		Monocyte	208 (138–315)	0.0002†
Anemia/cerebral‡ vs. neither	15	PMN	516 (239–394)	0.008†
		Monocyte	316 (230–802)	0.08
Hyperparasitemia vs. Pf < 500,000§	85	PMN	159 (97–221)	0.71
		Monocyte	522 (236–808)	0.04†
			318 (244–391)	
			707 (171–1,244)	
			199 (128–269)	
			428 (45–810)	
			334 (236–431)	
			364 (238–489)	
			143 (50–235)	
			290 (185–396)	

* CI = confidence interval.

† Denotes significance at $P \leq 0.05$.

‡ Implies concomitant severe anemia (hemoglobin ≤ 5 g/dL) and a cerebral malaria diagnosis.

§ Pf = *Plasmodium falciparum* parasite density/mm³.

a lower peripheral parasite density in children who died compared with children that survived, although the statistical significance of this finding disappeared upon removing hyperparasitemia as a criterion for severe disease. Children with hyperparasitemia alone are presumably healthier children who were more likely to survive. It is possible that this group should be considered separately from those with more severe forms of malaria, which would require larger studies, probably including multiple sites.

Ongoing trials conducted through the Severe Malaria in African Children network have a large sample size at multiple study sites and should further illuminate the significance of malaria pigment in severe malaria, including hyperparasitemia. Linear regression models taking into account hyperparasitemia or peripheral parasite density demonstrated no association with either pigmented PMNs or monocytes. An inconsistency was noted in that monocyte presence did not appear to be associated with hyperparasitemia on regression analysis, although upon stratifying patients with peripheral parasitemias greater than and less than 500,000 asexual forms/mm³, increased monocyte pigment was observed in patients without severe disease. While the significance of this finding is unclear, we have demonstrated that the degree of parasitemia is not associated with increased amount of pigment seen in leukocytes, consistent with our hypothesis that the presence of pigment reflects overall sequestered parasite burden.

In summary, in an age-, residence-, and ethnicity-matched case-control study, we have found malaria pigment/mm³ in neutrophils to be associated with disease mortality in Malian children three months to 14 years of age. We have validated previously reported associations between the amount of neutrophilic malaria pigment and disease severity, and have established an association with monocyte pigment when measured as pigment/mm³. In this study, we found that intra-neutrophilic pigment was associated with cerebral malaria and the combination of cerebral malaria and severe anemia, while intra-monocytic pigment is associated with severe anemia. These results do not appear to be biased by the degree of peripheral parasitemia. Based on these results, the assessment

of intraleukocytic pigment appears to be valuable for more detailed characterization of children who present with *P. falciparum* parasitemia and clinical symptoms consistent with a malarial illness.

Received April 29, 2003. Accepted for publication July 1, 2003.

Acknowledgments: We thank the members of the Severe Malaria in African Children Clinical Network for their work in the genesis of this study and the protocol to which we adhered. We also thank Timothy Mnalemba (Blantyre Malaria Project, Blantyre, Malawi) for his patient teaching, which was invaluable in identifying pigment and applying the protocols.

Financial support. This study was supported by contract no. N01-AI-85346 from the National Institutes of Health.

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