PIGMENTED MONOCYTES ARE NEGATIVE CORRELATES OF PROTECTION AGAINST SEVERE AND COMPLICATED MALARIA IN UGANDAN CHILDREN

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Abstract. Pigmented leukocytes are reported to be associated with severe malaria (SM). Blood smears from a case-control study of SM conducted in Apac Hospital in Northern Uganda were examined for pigmented leukocytes to investigate their association with measures of disease and clinical immunity in children less than 5 years old. Pigmented leukocytes, predominated by monocytes, were significantly greater in number in SM by comparison with uncomplicated malaria (UM). SM children with no pigmented leukocytes had significantly elevated hemoglobin, packed cell volumes, and titers of IgG anti-SERA5 by comparison with SM children with pigmented leukocytes. These differences were not observed in UM. A Spearman rank correlation analysis showed, in addition, a negative but weak correlation between pigmented monocytes and titers of IgG anti-Plasmodium falciparum lysate and IgG anti-EBA-175 in both SM and UM children. Thus, numbers of pigmented monocytes might be negative correlates of clinical immunity in a region of holoendemic malaria.

INTRODUCTION

Hemoglobin catabolism is switched on during the ring stage in the food vacuole of Plasmodium falciparum where FeIII-porphyrin, a by-product of hemoglobin digestion, is incorporated into β-hematin, the principal pigment of hemozoin.1 This process is inhibited by chloroquine in chloroquine-susceptible P. falciparum strains.1,2 Hemozoin consists of FeIII-porphyrin units linked by propionate oxygen-iron bonds into head-to-tail dimers; the insoluble crystal is released into circulation and engulfed by phagocytes such as monocytes and neutrophils.3–5 Pigmented leukocytes can be easily discerned under the light microscope as a black, brown, or amber pigment.7 Several studies have reported the usefulness of enumerating pigmented leukocytes as a measure of malaria disease severity and prognosis in African children and Thai adults.8–12 There is also increasing evidence that hemozoin might mediate immunosuppression during malaria through a number of mechanisms.13–16 Our working hypothesis was that the number of pigmented leukocytes in blood smears of pediatric malaria patients might be inversely correlated with the level of clinical immunity in individual children. In this paper we report our studies that not only confirmed the previous reports of an association between pigmented leukocytes and severe and complicated malaria in African children but also showed that numbers of pigmented monocytes showed a negative correlation with levels of hemoglobin, packed cell volume, and proxy measures of protection against severe malaria in Ugandan children.

MATERIALS AND METHODS

Study design and study population. The study site was located in a region of intense holoendemic malaria transmission in Northern Uganda.17 Two hundred eight infants were recruited in a case-control study of severe malaria conducted at Apac Hospital in Apac Town; the demographic and clinical details of the study subjects have been reported in detail elsewhere.18 Briefly, ethical approval was obtained from the Uganda National Council for Science and Technology, and parental consent was obtained before the enrollment of the children and blood collection. The study population were children 6–59 months old who were attending or admitted to Apac Hospital with a primary diagnosis of malaria. A standard questionnaire on the clinical history and the use of anti-malarial drugs and protective anti-mosquito measures was filled out, followed by a physical examination by a physician. The criteria for severe and complicated malaria versus uncomplicated or mild malaria were according to the World Health Organization (WHO) definition.19 The conditions considered in the differential diagnosis of severe malaria were septicemia, typhoid fever, pyelonephritis, lobar pneumonia, and viral hepatitis. The control children, matched for age, sex, and geographic location or parish residence, were recruited from the Outpatient Department when they presented with other illnesses and were well enough to go home. The exclusion criteria included age less than 6 months or more than 60 months, presentation with life-threatening illnesses, clinical signs of pneumonia, bacterial or parasite-related gastroenteritis, HIV-1/AIDS–related opportunistic infections, or parental refusal to provide informed consent.

Laboratory analyses. Two-microliter venous blood samples were collected from both cases and controls; serum samples were stored at −80°C until analyzed. Hemoglobin and packed cell volume (PCV) were measured colorimetrically and using a hematocrit centrifuge, respectively. Thick and thin blood smears were prepared and Giemsa-stained for microscopic confirmation of P. falciparum parasites in the blood. A rapid examination of thick films was carried out for clinical diagnosis of malaria at the hospital so that children with confirmed P. falciparum infections received prompt treatment with anti-malarial drugs according to the national guidelines. Thin films were examined in detail in Kampala for the enumeration of parasite density and pigmented leukocytes. A total of 500 leukocytes were counted for each slide while scoring the total number of pigmented leukocytes (both monocytes and neutrophils), pigmented monocytes, or pigmented neutrophils on a differential cell counter. The estimated number of pigmented leukocytes, monocytes, and neutrophils per microliter of blood was calculated by multiplying the respective values by 16 (8,000/500). This is based on the assumption that 1 μL blood contains 8,000 leukocytes.20 Ti-
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ters of serum IgG antibodies against a total \textit{P. falciparum} extract and specific IgG antibodies against recombinant constructs of EBA-175 and SERA5 were measured by enzyme-linked immunosorbent assay as described.\textsuperscript{18}

\textbf{Statistical analysis.} EPI-Info-6 program (CDC Atlanta, Epidemiology Program Office, Atlanta, GA) was used for data entry. Statistical analyses were carried out using the Microsoft Excel-based program Analyze-It (Analyze-It Software, Leeds, UK). The median and interquartile range (IQR) of variables were compared using the non-parametric Mann-Whitney \textit{U} test. Correlations between variables were assessed by the Spearman rank correlation coefficient. To carry out \textit{χ}\textsuperscript{2} tests for the association between pigmented leukocytes and several parameters, severe malaria (SM) and uncomplicated malaria (UM) children were sub-divided into two groups based on hemoglobin (1–9 versus ≥ 10 g/dL), PCV (≥ 29 versus ≥ 30%) levels, and titers of specific antibodies (low versus high titers). Low and high titers for IgG anti-SERA5, IgG anti-\textit{P. falciparum}, and IgG anti-EBA-175 were 1/250–1/1,000 versus ≥ 1/1,000, 1/250–1/4,000 versus ≥ 1/8,000, and 1/250 versus ≥ 1/250, respectively.

\section*{RESULTS}

\textbf{Clinical characteristics of the study population.} The details of the demographic, clinical, and laboratory findings of the case-control study of severe malaria in children less than 5 years of age presenting at Apac Hospital in Northern Uganda have been reported.\textsuperscript{18} The most common presentations of SM in hospitalized children were respiratory distress (53.4%) and prostration (40.4%), followed by circulatory collapse (7.4%), severe anemia (hemoglobin < 5 g/dL; 7.0%), and seizures (2.6%). No cases of impaired consciousness, coma, or cerebral malaria were observed among the SM children in this study.

\textbf{Quantification of pigmented leukocytes.} The range and median (IQR) numbers of pigmented leukocytes, pigmented monocytes, and pigmented neutrophils per microliter of blood and the proportion of children with pigmented leukocytes in blood smears are presented in Table 1. These measures of pigmented leukocytes were all significantly greater in SM children compared with UM children. In SM and UM blood smears, the total estimated number of pigmented monocytes as a proportion of the total number of pigmented leukocytes per microliter of blood was 3,852/8,832 (94.6%) and 2,080/2,096 (99.2%), respectively. In contrast, the total estimated number of pigmented neutrophils as a proportion of the total number of pigmented leukocytes per microliter of blood in SM and UM blood smears was 480/8,832 (5.4%) and 16/2,096 (0.80%), respectively. Pigmented neutrophils were observed only in the presence of pigmented monocytes. These data clearly showed the predominance of pigmented monocytes and the paucity of pigmented neutrophils in the peripheral blood smears of our pediatric study population.

\textbf{Measures of disease or immunity and numbers of pigmented leukocytes.} To assess the relationship between pigmented leukocytes and proxy measures of disease (hemoglobin, PCV, temperature, parasitemia) or immunity (age, titers of specific IgG antibodies against \textit{P. falciparum} lysate, SERA5, and EBA-175), the children were sub-divided into two groups, namely, those with and those without pigmented leukocytes (Table 2). SM children with no pigmented leukocytes had significantly higher median hemoglobin, PCV, and titers of IgG anti-SERA5 by comparison with their counterparts with pigmented leukocytes; the other parameters were comparable between the two groups. In contrast, UM children with no pigmented leukocytes were significantly older and had comparable measures of morbidity and immunity in comparison with UM children with pigmented leukocytes. Because having pigmented leukocytes was associated with significantly lower hemoglobin and PCV values and proxy measures of immunity in SM children, we arbitrarily subdivided SM children into those with low (≤ 150) and high (> 150) numbers of pigmented leukocytes to establish if there was a dose-effect of the pigment on measures of disease or immunity. SM children with high numbers of pigmented leukocytes had significantly reduced hemoglobin, PCV, and IgG anti-\textit{P. falciparum} lysate in comparison with those with low numbers of pigmented leukocytes (\textit{P} = 0.004, 0.01, and 0.04, respectively); the other measures of morbidity and immunity were comparable. Figures 1 and 2 show the dose-effect of pigmented monocytes on median hemoglobin and titers of IgG anti-SERA5, IgG anti-EBA-175, and IgG anti-\textit{P. falciparum} lysate in SM children.

We next determined the correlation between numbers of pigmented monocytes and specific measures of disease and immunity (Table 3). In SM children, there was a highly significant negative correlation between numbers of pigmented monocytes, on one hand, and hemoglobin, PCV, and IgG anti-SERA5 on the other; there was a weak but significant negative correlation with titers of IgG anti-\textit{P. falciparum} lysate and IgG anti-EBA-175 but no significant correlation with other parameters. In UM children, there was a highly significant negative correlation between numbers of pigmented monocytes and age, and a weak but significant negative correlation with IgG anti-\textit{P. falciparum} lysate and IgG anti-

\begin{table}[h]
\centering
\caption{Measures of pigmented leukocytes in Ugandan children with severe or uncomplicated malaria\footnote{The values are the proportions (%) of blood smears with pigmented monocytes or neutrophils.}}
\begin{tabular}{llllllll}
\hline
\textbf{Parameter} & \textbf{Severe malaria (N = 99)} & & \textbf{Uncomplicated malaria (N = 96)} & & \textbf{Statistic} & \textbf{P} \\
\hline
\textbf{Age (months)} & 6–59 & 13.0 (10.8) & & 6–59 & 13.0 (13.0) & 4634.0 & 0.9567 \\
\textbf{Total pigmented WBC} & 0–640 & 32.0 (128.0) & & 0–272 & 0.0 (28.0) & 6312.0 & < 0.0001 \\
\textbf{No. pigmented monocytes} & 0–640 & 32.0 (126.5) & & 0–272 & 0.0 (1.00) & 6752.0 & < 0.0001 \\
\textbf{Pigmented monocytes} & & & & & & 17.8 & < 0.0001 \\
\textbf{No. pigmented neutrophils} & 0–80 & 0.0 (0.0) & & 0–16 & 0.0 (0.0) & 5040.0 & 0.0002 \\
\textbf{Pigmented neutrophils} & 16/99 (16.2) & & 1/96 (1.0) & 12.2 & 0.0005 \\
\hline
\end{tabular}
\end{table}
EBA-175. The results of these analyses were confirmed by χ² tests for association. There was a significant association between the presence of pigmented monocytes and the malaria clinical outcome in general (χ² = 17.81; P < 0.0001). In SM children, there was a significant association between the presence of pigmented monocytes on one hand, and hemoglobin (χ² = 7.53, P = 0.006) and titers of IgG anti-SERA5 (χ² = 17.29, P < 0.0001) on the other. However, as for the correlation analysis, there was no significant association between the presence of pigmented monocytes and age in SM children (χ² = 1.44, P = 0.23); in UM children, the only significant association was that between the presence of pigmented monocytes and age (χ² = 5.18, P = 0.023).

Finally, after adjusting for the effect of age and titers of IgG anti-EBA-175 and IgG anti-*P. falciparum* lysate in a multiple logistic regression model (Intercooled Stata for Windows, version 8.2; Stata Corp., College Station, TX), the odds ratio (OR) of having pigmented monocytes per unit increase in the titer of IgG anti-SERA5 was significant in SM children (OR, 0.018; SE, 0.032; z = −2.26; P = 0.024) but not in UM children (OR, 2.64; SE, 1.61; z = 1.59; P = 0.111). When the effect of age and titer of IgG anti-SERA5 were adjusted for, the OR of having pigmented monocytes per unit increase in the titer of IgG anti-EBA-175 was not significant in SM children (OR, 0.69; SE, 0.361; z = −0.71; P = 0.477) but was significant in UM children (OR, 0.386; SE, 0.160; z = −2.29; P = 0.022). For both SM and UM children, the OR of having pigmented monocytes per unit increase in the titer of IgG anti-*P. falciparum* lysate was not significant (P = 0.887 and 0.327, respectively). These data suggest that high titers of IgG anti-SERA5 and IgG anti-EBA-175 antibodies are significantly associated with a reduced OR of having pigmented monocytes in SM and UM children, respectively.

Although pigmented neutrophils were observed in only 19/99 (16.2%) of SM children, median (IQR) hemoglobin levels were significantly higher in SM children without pigmented neutrophils in comparison with those in SM children with pigmented neutrophils (8.2 [3.7] g/dL, N = 16 versus 7.0 [2.8] g/dL, N = 83; Mann-Whitney U = 406; P = 0.014) and PCV (23.8% [9.3%], N = 15 versus 20.3% [6.45%], N = 79; Mann-Whitney U = 313; P = 0.004). A correlation analysis showed a significant inverse correlation between numbers of pigmented neutrophils and hemoglobin (Spearman rs = −0.23; P = 0.02) as well as PCV (Spearman rs = −0.28; P = 0.006) in SM children. In view of the paucity of pigmented neutrophils in UM children (1/96 [1.04%]), the analysis of the correlation between pigmented neutrophils and measures of morbidity or immunity were not carried out in this group.

**DISCUSSION**

Previous studies of pigmented leukocytes in African infants and Thai adults have shown an association between numbers
of pigmented leukocytes and the severity and prognosis of severe and complicated malaria, specifically between pigmented neutrophils and death and cerebral malaria on one hand, and between pigmented monocytes and severe anemia on the other. In this study, we observed a significant association between the presence of pigmented leukocytes in peripheral blood smears, predominated by monocytes, and the clinical manifestation of malaria in Ugandan children less than 5 years who were residents in a holoendemic region in Northern Uganda. In these children, all measures of pigmented leukocytes were significantly higher in SM children compared with UM children. Our study provided corroborative evidence that, in SM children, pigmented monocytes are associated, in a dose-dependent manner, with the lower hemoglobin seen in malaria anemia. Pigmented neutrophils were conspicuously rare in both UM and SM children. The reason for the paucity of pigmented neutrophils in this study population is not clear. A previous study suggested that the presence of pigmented neutrophils might be a reflection of a more severe and fulminant course of disease, which ends in death, whereas the presence of pigmented monocytes indicates a more chronic or indolent infection.

There is evidence that the risk of severe disease in childhood malaria is lowest among populations with the highest transmission intensity. This finding suggests that the quantity of pigmented leukocytes is probably a reflection of the total parasite biomass sequestered in tissues rather than peripheral blood parasitemia per se.

There is evidence that clinical immunity against severe malaria increases with age, probably as a result of the cumulative exposure to P. falciparum infections. We have previously shown in the same pediatric population that high titers of IgG anti-SERA5 are associated with protection against SM. IgG anti-Pf lysate, IgG anti-SERA5; and IgG anti-EBA-175 were dynamically associated with protection against SM. The number of missing data points in SM and CM children were 1 and 2, respectively.

### Table 3

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Spearman’s correlation</th>
<th>N</th>
<th>rs</th>
<th>95% CI</th>
<th>P</th>
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<td>Packed cell volume</td>
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<td>-0.51 to -0.16</td>
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<td>-0.46 to -0.09</td>
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</table>

*The values are sample sizes (N). Spearman’s correlation (rs), and 95% confidence interval (95% CI). The values in bold indicate statistically significant P values.

†The number of missing data points in SM and CM children were 1–14 and 1–20, respectively.

Pf, P. falciparum

**Figure 2.** Median titers of serum IgG anti-P. falciparum lysate (top), IgG anti-SERA5 (middle), and IgG anti-EBA-175 (bottom) in SM children with no pigmented monocytes (open bars) and SM children with pigmented monocytes (hatched bars). The IQRs for median specific antibody titers in the 0, <150, and >150 groups are 7000, 6500, and 1500 for IgG anti-P. falciparum lysate; 1000, 500, and 500 for IgG anti-SERA5; and 375, 250, and 250 for IgG anti-EBA-175.
antibodies against EBA-175 region II, one of the antigens used in this study, inhibit erythrocyte invasion.\textsuperscript{26} Titers of IgG anti-EBA-175 antibodies are associated with protection against severe malaria in our pediatric study population (Magambo et al., unpublished observations). We also made the assumption that IgG anti-\textit{P. falciparum} lysate might contain antibody specificities that are associated with protection against severe malaria. In this study, we therefore used age, IgG anti-SERA5, IgG anti-EBA-175, and IgG anti-\textit{P. falciparum} lysate as proxy measures of clinical immunity. SM children with no pigment leukocytes had significantly higher titers of IgG anti-SERA5, and the numbers of pigmented leukocytes in SM children were inversely correlated with titers of IgG anti-SERA5. In UM children, there was a negative correlation between numbers of pigment leukocytes on one hand, and age on the other. Multiple logistic regression analyses confirmed that titers of IgG anti-SERA5 and IgG anti-EBA-175 were associated with a significantly reduced occurrence of pigment leukocytes in SM and UM, respectively. The differential results of the multiple logistic regression and the effect of age on numbers of pigment leukocytes in SM and UM probably reflect the different mechanisms of immunity mediated by IgG anti-SERA5 and IgG anti-EBA-175 in the two different manifestations of malaria. The attrition or clearance of pigment leukocytes seems to be associated with both mechanisms. These observations provided strong evidence that the presence and number of pigment monocytes are negative correlates of protection against severe malaria in Ugandan children less than 5 years old who are residents in a region of holoendemic malaria. These microscopic findings differ from those obtained by flow cytometry, which showed an increased frequency of pigment monocytes in semi-immune adult Europeans with malaria.\textsuperscript{27} There is increasing evidence that \textit{P. falciparum} hemozoin mediates immunosuppression through a number of mechanisms. First, it modulates the cytokine responses of human peripheral blood mononuclear cells \textit{in vitro} in a dose-dependent manner, with a drastic decrease in interleukin (IL)-2, IL-12, and interferon (IFN)-\gamma levels, whereas IL-10 levels increase with the hemozoin load of monocytes/macrophages in culture.\textsuperscript{13} Second, \textit{in vivo} acquisition of high levels of hemozoin by placental mononuclear cells leads to a decreased synthesis of prostaglandin-E2, IL-10, and tumor necrosis factor (TNF)-\alpha.\textsuperscript{14} Third, hemozoin inhibits the differentiation and maturation of monocyte-derived dendritic cells, which probably contributes to the impairment of monocyte-mediated immune functions.\textsuperscript{15} Finally, a case-control study of severe malaria in Gabonese children reported that IL-12 levels showed an inverse correlation, whereas acute phase TNF-\alpha and IL-10 plasma levels showed a positive correlation with numbers of circulating pigment neutrophils.\textsuperscript{16} These published findings probably explain the observed negative correlation between numbers of pigment monocytes and putative measures of clinical immunity in Ugandan children.

In summary, our study confirmed previous reports about the association between pigment leukocytes and severe malaria, specifically the association between pigment monocytes and anemia. The study also provided additional evidence, for the first time, about the inverse correlation between pigment monocytes and putative proxy measures of clinical immunity in Ugandan children less than 5 years old. Pigmented monocytes are simple to score by ordinary light microscopy, and the data can be easily generated, because the same slides used for malaria microscopy can be used for the enumeration of pigment cells. Measurements of pigmented monocytes might be useful endpoints in malaria vaccine trials in African children.

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