The Frequency of BDCA3-Positive Dendritic Cells Is Increased in the Peripheral Circulation of Kenyan Children with Severe Malaria

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Received 31 May 2006/Returned for modification 12 July 2006/Accepted 16 September 2006

The ability of Plasmodium falciparum-infected erythrocytes to adhere to host endothelial cells via receptor molecules such as ICAM-1 and CD36 is considered a hallmark for the development of severe malaria syndromes. These molecules are also expressed on leukocytes such as dendritic cells. Dendritic cells are antigen-presenting cells that are crucial for the initiation of adaptive immune responses. In many human diseases, their frequency and function is perturbed. We analyzed the frequency of peripheral blood dendritic cell subsets and the plasma concentrations of interleukin-10 (IL-10) and IL-12 in Kenyan children with severe malaria and during convalescence and related these parameters to the adhesion phenotype of the acute parasite isolates. The frequency of CD1c+ dendritic cells in children with acute malaria was comparable to that in healthy controls, but the frequency of BDCA3+ dendritic cells was significantly increased. Analysis of the adhesion phenotypes of parasite isolates revealed that adhesion to ICAM-1 was associated with the frequency of peripheral blood CD1c+ dendritic cells, whereas the adhesion of infected erythrocytes to CD36 correlated with high concentrations of IL-10 and low concentrations of IL-12 in plasma.

In areas of endemicity, infection with asexual blood stages of Plasmodium falciparum can result in asymptomatic infection, mild clinical symptoms, or severe, life-threatening disease (1). Clinical immunity against asexual blood-stage infection is never sterile but is built up with repeated exposure, eventually allowing mature forms to sequester in the vascular bed, leading to the obstruction of capillary vessels. In particular, adhesion promotes chronic infection (4). Importantly, PFEEMP-1 also mediates adhesion of mature forms of infected red blood cells (iRBCs) to host proteins expressed on endothelial cells and leukocytes. Although almost all parasite isolates adhere to CD36, some also binds to other receptors such as CD54 (ICAM-1 [intercellular adhesion molecule 1]), CD31 (PECAM-1 [platelet endothelial cell adhesion molecule 1]) or CD35 (CR1 [complement receptor 1]) (18). Cytoskeletal rearrangements detected that have distinct but overlapping functions. Myeloid DCs (mDCs) express HLA DR, CD11c and CD1c and are the main producers of interleukin-12 (IL-12), whereas plasmacytoid DCs (pDCs) express HLA DR, CD123, and BDCA2 (blood dendritic cell antigen 2) and are the main producers of IFN-α. A third, minor population of CD11c+ BDCA3+ mDCs in peripheral blood has been described but is not well characterized (9). In vitro studies on monocyte-derived DCs suggested that adhesion of iRBCs to surface-expressed CD36 modulated both their maturation and function (32). In these studies, parasite-modulated DCs failed to secrete IL-12 or to induce proliferation in naive or primed T cells, although they secreted IL-10 and tumor necrosis factor alpha (TNF-α).

We have previously reported that the frequency of total peripheral blood DCs remained constant during acute falciparum malaria, whereas HLA DR expression was reduced, suggesting that modulation of DCs may occur in vivo (33). Furthermore, a recent study by Pichyangkul et al. showed that the frequency of pDCs in peripheral blood was reduced in adult Thai patients with acute malaria (26). We now wanted to establish whether changes in DC numbers and the expression of HLA DR were similar for all subsets in Kenyan children with severe malaria or whether these phenomena are different for each subset. Therefore, we investigated changes in the frequency of specific DC subsets in Kenyan children with severe malaria in acute and convalescent samples compared to healthy community controls. In addition, we analyzed whether there is any relationship between the frequency

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Published ahead of print on 25 September 2006.
of peripheral blood DC subsets, the concentration of key cytokines in plasma, and the adhesion phenotype of the acute parasite isolate.

MATERIALS AND METHODS

Study population. Blood samples were collected from children presenting to Kilifi District Hospital on the coast of Kenya with severe P. falciparum malaria. Severe malaria was characterized by the presence of one or more of the following features: signs of deep breathing, coma (Blantyre coma score of ≥2), prostration, or severe anemia (hemoglobin [Hb] < 5 g/dl) in the presence of hyperparasitemia (iRBC > 10%). Children were excluded if they showed any sign of bacterial or viral meningitis, including positive blood or cerebrospinal fluid cultures or white blood cells in the cerebrospinal fluid. Children were invited for convalescent sampling 14 days after discharge from hospital, at which time they were examined clinically and treated if necessary. Children who were still slide positive for parasites were excluded from the analysis. Control blood samples were collected from children living in the Ngereya area of Kilifi District, who were part of a cohort under active surveillance for malaria as described in detail elsewhere (23). These children were sampled during a cross-sectional survey conducted during a period of low transmission in October 2004. Children who were slide positive for parasites or had a temperature ≥38°C on admission were treated if necessary. Children who were still slide positive for parasites were excluded from further analysis.

Flow cytometry. Venous blood samples (500 μl) were drawn into EDTA blood tubes (TekLab, United Kingdom), and 50-μl aliquots were incubated for 30 min at 4°C with a cocktail containing the lineage markers antibodies (anti-CD3, anti-CD14, anti-CD19, anti-CD11c, anti-CD1d, and anti-CD1c; BDCA2, BDCA3, and BDCA4; PE-conjugated anti-CD3, anti-CD14, and anti-CD19; PC5-conjugated anti-HLA DR and R-phycoerythrin-conjugated anti-CD14; Dako, United Kingdom) and 50-μl aliquots were incubated for 30 min at 4°C with anti-CD14 and anti-HLA DR. After erythrocyte lysis with OptiLyse C solution (Beckman Coulter, United Kingdom), white blood cells were washed in phosphate-buffered saline and analyzed by flow cytometry (Epics II; Beckman Coulter, United Kingdom). For each sample, we acquired at least 1,000,000 events per sample. The percentage of CD1c+ cells in the monocyte population was determined by whole-blood staining (Fig. 1). The absolute numbers of CD1c+ or BDCA3+ mDCs, pDC, and monocytes were calculated by using whole-blood counts. All flow cytometry data were analyzed by using FlowJo software (TriStar).

Enzyme-linked immunosorbent assay. Plasma and PBMC were separated by density centrifugation using Lymphoprep according to standard methods, and plasma was frozen immediately at −80°C. The concentrations of IL-10, TNF-α, and IL-12 in plasma were analyzed by using proprietary ELISA kits (Pharmin-gen) according to the manufacturer’s instructions.

Parasite culture and binding assay. RBCs from acute blood samples were taken into culture and parasites grown to maturity in the presence of phagocidal (Sigma), a reversible DNA synthesis inhibitor which arrests parasite development during the trophozoite stages. Presence of phagocytosis does not affect agglutination, rosetting or adhesion to ICAM-1 and CD36 (9, 13). Parasitemia and hematocrit were determined by flow cytometry after staining iRBCs with ethidium bromide. Cultures with a parasitemia above 3% were used for binding assays as previously described (22). Briefly, 2 μl each of 100, 20, 4, or 0.8 μg of iCAM-Fc (8) or purified human immunoglobulins/ml and 50, 10, 2, or 0.4 μg of CD36-Fc (R&D Systems, United Kingdom) or CD31-Fc (10)/ml were absorbed onto plastic bacteriological dishes. Parasite cultures were adjusted to 1% hematocrit in binding medium and then added to the plastic dish. Parasites were incubated for 1 h at 37°C with gentle rotation every 10 min. Nonadherent cells were washed away, and adherent cells were fixed with 1% glutaraldehyde (Sigma, United Kingdom) and then stained with Giemsa (Sigma, United Kingdom). The number of adherent parasites in three different sections of each protein spot were counted by using light microscopy (magnification, ×400) and corrected to the number of cells bound per square millimeter of protein at 2% parasitemia and 1% hematocrit. At the highest protein concentration, the average standard deviation for binding to ICAM-1 was ±7 iRBC/mm², and the average standard deviation for binding to CD36 was ±11 iRBC/mm².

RESULTS

Frequency of peripheral blood DC subsets. Of the 65 children with severe malaria recruited into the study, 33 returned to give a convalescent blood sample on day 14. As expected, children with severe malaria had significantly lower RBC counts and hemoglobin concentrations compared to controls. While these parameters had improved in convalescence, they had not yet returned to normal (Table 1).

We analyzed the absolute number of peripheral blood DC subsets and monocytes in paired samples from children with severe malaria and during convalescence and in the control population by whole-blood staining (Fig. 1). The absolute number of myeloid CD1c+ mDCs and pDCs remained relatively constant during severe malaria and convalescence (Table 2). In contrast, the absolute number of BDCA3+ mDCs and monocytes was significantly increased in children with severe malaria versus controls (Table 2).

We observed previously that the expression of HELA DR was reduced on peripheral blood DCs, suggesting functional impairment of DCs, although in that study we were not able to distinguish mDCs and pDCs. We confirm here that expression of HELA DR was reduced on monocytes and CD1c+ and BDCA3+ mDC but not on pDC when acute and convalescent samples (Wilcoxon signed-rank test for paired acute and convalescence samples. The concentrations of IL-10, TNF-α, and IL-12 in plasma, the number of iRBCs binding to immobilized protein, and the number of peripheral blood DC subsets and monocytes were normally distributed after logarithmic transformation. Logarithmic transformed values were used to obtain correlation coefficients between these parameters within one group and for linear regression analysis. All P values are two sided.

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Plasma cytokine concentrations. We determined the plasma concentrations of TNF-α, IL-12, and IL-10, all of which are produced by parasite-modulated DCs in vitro, to investigate whether any of these were associated with the frequency of DC subsets. All cytokines were significantly increased during acute disease compared to convalescent and control samples (Table 2), whereas IL-10 was also detectable in plasma during convalescence and in some healthy children. In children with acute disease, the concentrations of TNF-α, IL-12, and IL-10 in plasma did not correlate with each other. The plasma concentration of IL-10 was positively correlated with parasitemia (14) and the number of BDCA3+ mDCs in children with acute malaria (Pearson correlation coefficient: parasitemia, \( r = 0.794, P < 0.001 \); BDCA3+ mDC, \( r = 0.393, P < 0.05 \)). The concentration of IL-12 or TNF-α in plasma did not correlate with the absolute number of or HLA DR expression on any of the DC subsets or monocytes.

### Antigen-presenting function of PBMC

Chehimi et al. had reported an association between DC frequencies in PBMC and their ability to stimulate proliferation of allogeneic T cells (6). We used this test to investigate whether the frequency of DC subsets or the expression level of HLA DR in PBMC from children with severe disease, from children during convalescence, and from controls was related to their ability to induce proliferation in allogeneic T cells. PBMC from children with severe malaria induced less T-cell proliferation than PBMC from healthy children irrespective of the PBMC/T-cell ratio used (Fig. 2, \( P < 0.01 \) [Mann-Whitney U test]) at a PBMC/T-cell ratio of 1:1 and 1:2). The extent of T-cell proliferation was negatively correlated with the number of BDCA3+ mDC (Spearman rho \(-0.363 [P = 0.035] \) and rho \(-0.352 [P = 0.046] \) for PBMC/T-cell ratios of 1:2 and 1:1, respectively) during acute disease, and a similar trend was observed for the convalescence group. Unlike the observation by Chehimi et al., we found no correlation among the control population between the DC frequency in PBMC and the induction of proliferation in T cells.

### Adhesion phenotypes of homologous parasite isolates

We were interested to see whether the adhesion phenotype of the acute parasite isolate correlated with the frequency of peripheral blood DCs or the plasma concentrations of IL-10, IL-12, or TNF-α. We therefore determined the binding to plate-bound CD36, ICAM-1, CD31, and human immunoglobulin for all parasite isolates, which grew to maturity (\( n = 26 \)), whether or not the child returned for a follow-up appointment. All isolates bound to CD36 and to ICAM-1 and, in general, more trophozoites bound to CD36 at the highest concentration tested than to ICAM-1; the median (25th to 75th percentile) values for 50 \( \mu \)g of CD36/ml and 100 \( \mu \)g of ICAM-1/ml were 3,412 iRBC/mm² (2,650 to 5,156) and 759 iRBC/mm² (428 to 1,429), respectively (Fig. 3). Binding to CD36 was weak and above background levels only for two isolates, as has been observed in one study but not another study on Kenyan parasite isolates (7, 22). We did not observe any binding to human immunoglobulin, although we cannot exclude that immunoglobulin carried over from culture medium was bound to the surface of iRBC and blocked binding to plate-bound immunoglobulin (7). At concentrations of 100 \( \mu \)g of ICAM-1/ml and of 10 \( \mu \)g of CD36/ml, the numbers of iRBCs bound to ICAM-1 and to CD36 were comparable. At this concentration, the binding of iRBCs to CD36 was correlated with high plasma concentrations of IL-12 (Fig. 4) but not with the plasma concentration of TNF-α or the absolute number of any DC subset. Similar relationships were observed when we analyzed the binding of iRBC to 50 or 2 \( \mu \)g of plate-bound CD36/ml (Pearson correlation coefficients: for 50 \( \mu \)g of CD36/ml, \( r = 0.575, P = 0.04 \) [IL-10], and \( r = -0.557, P = 0.031 \) [IL-12]; for 2 \( \mu \)g of CD36/ml, \( r = 0.862, P = 0.013 \) [IL-10] and \( r = -0.416, P = 0.035 \) [IL-12]), although the binding of iRBCs to plate-bound CD36 at a concentration

![Flow cytometric analysis of peripheral blood DC subsets.](image_url)
of 50 μg/ml was outside the linear range. As described above, the concentration of IL-10 in plasma was also correlated with parasitemia. We therefore used multiple regression to determine the individual contributions of parasitemia and adhesion to CD36. In this model, both parameters were independently associated with the concentration of IL-10 in plasma (overall model $r^2 = 0.717$, $P < 0.001$; standardized beta coefficient of 0.42, $P = 0.019$ for parasitemia and 0.496, $P = 0.007$ for adhesion to CD36). In contrast, the binding of iRBCs to 50 μg of plate-bound ICAM-1/ml showed a positive correlation with the number of CD1c$^+$ mDCs and pDCs (Fig. 4) but not with the concentration of IL-10, IL-12, or TNF-α in plasma. Again, these correlations were also observed at the lower concentration of 10 μg of plate-bound ICAM-1/ml (Pearson correlation coefficient for CD1c$^+$ DC: $r = 0.572$, $P = 0.008$; Pearson correlation coefficient for BDCA2$^+$ DC: $r = 0.466$, $P = 0.038$).

**DISCUSSION**

Here we have shown that severe infection with *P. falciparum* malaria induces profound changes in the number of BDCA3$^+$ mDCs in the peripheral circulation, whereas the overall number of CD1c$^+$ mDCs and pDCs was not significantly altered during acute disease compared to convalescence samples or controls.

To our knowledge, this is the first report showing increased frequency of BDCA3$^+$ mDCs in a human infection. The function of this rather small population in healthy individuals is not known. However, BDCA3, together with other inhibitory receptors, is upregulated on IL-10-treated monocyte-derived DCs. IL-10-treated monocyte-derived DCs express intermediate levels of HLA DR and costimulatory molecules and fail to activate T cells (34), a phenotype similar to that of parasite-modulated monocyte-derived DCs. An immunomodulatory function of this DC subset is in agreement with our observation that reduced allogeneic T-cell proliferation induced by PBMC from children with acute malaria was associated with an increased frequency of BDCA3$^+$ mDCs. It has recently been suggested that CD1c$^+$ and BDCA3$^+$ mDC subsets represent different stages of mDCs rather than different lineages because their transcription profile is very similar (21). We observed an

### TABLE 2. DC numbers and cytokine concentrations in cases and controls

<table>
<thead>
<tr>
<th>Parameter $^a$</th>
<th>Median (25th–75th percentile)$^b$</th>
<th>Acute sample $(n = 33)$</th>
<th>Convalescent sample $(n = 33)$</th>
<th>Control sample $(n = 33)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD1c$^+$ mDCs (10$^3$/ml)</td>
<td>9.5 (5.8–18.9)</td>
<td>15 (8.8–26.5)</td>
<td>13 (9.3–18.5)</td>
<td></td>
</tr>
<tr>
<td>CD123$^+$ pDCs (10$^3$/ml)</td>
<td>4.9 (2.3–13.1)</td>
<td>9.4 (5.3–20.8)</td>
<td>9.3 (4.6–13.8)</td>
<td></td>
</tr>
<tr>
<td>BDCA3$^+$ mDCs (10$^3$/ml)</td>
<td>5.0$^*$ (2.1–9.0)</td>
<td>6.6$^*$ (2.1–30.0)</td>
<td>1.2 (0.6–2.0)</td>
<td></td>
</tr>
<tr>
<td>CD14$^+$ monocytes (10$^6$/ml)</td>
<td>0.39$^*$ (0.19–0.73)</td>
<td>0.51$^*$ (0.26–0.93)</td>
<td>0.10 (0.08–0.14)</td>
<td></td>
</tr>
<tr>
<td>CD1c$^+$ mDCs (GMFI HLA DR)</td>
<td>24.3$^*$ (17.0–34.2)</td>
<td>41.4 (28.3–49.6)</td>
<td>36.5 (30.3–44.3)</td>
<td></td>
</tr>
<tr>
<td>CD123$^+$ pDCs (GMFI HLA DR)</td>
<td>12.6 (6.6–21.1)</td>
<td>15.1 (10.8–19.3)</td>
<td>16.9 (11.5–20.1)</td>
<td></td>
</tr>
<tr>
<td>BDCA3$^+$ mDCs (GMFI HLA DR)</td>
<td>9.2$^*$ (5.1–15.0)</td>
<td>24.1 (15.8–37.5)</td>
<td>19.7 (13.4–28.8)</td>
<td></td>
</tr>
<tr>
<td>CD14$^+$ monocytes (GMFI HLA DR)</td>
<td>13.6$^*$ (10.1–24.1)</td>
<td>27.5 (17.8–52.0)</td>
<td>31.5 (28.3–36.5)</td>
<td></td>
</tr>
<tr>
<td>IL-10 (pg/ml)</td>
<td>1,731$^*$ (538–2,533)</td>
<td>69 (21–140)</td>
<td>44 (17–82)</td>
<td></td>
</tr>
<tr>
<td>TNF-α (pg/ml)</td>
<td>35$^*$ (11–82)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>IL-12 (pg/ml)</td>
<td>183$^*$ (91–274)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ GMFI HLA DR, Geometric mean fluorescence intensity of HLA DR expressed on monocytes or DC subsets. Of note, the fluorescence intensity scale on an Epics II flow cytometer from Beckman Coulter begins with 0.1 rather than 1.

$^b$ *, $P < 0.001$ compared to control (Mann-Whitney U test).
increase in the expression of BDCA3 on CD1c+ mDCs cocultured with iRBC in vitro (our unpublished observation), suggesting that the increased number of BDCA3+ mDCs may be due in part to the induction of BDCA3 expression on a proportion of CD1c+ DCs in children with acute malaria. We are now investigating the function of CD1c+ and BDCA3+ mDCs cocultured with iRBCs in vitro.

In addition to BDCA3+ mDCs, the number of monocytes was increased in the peripheral circulation, whereas the number of CD1c+ mDCs and pDCs remained constant. In a previous study, we reported that the total number of HLA DR+, lineage marker-negative DCs, comprising all three subsets, was apparently reduced due to the lower expression of HLA DR on DCs in children with acute malaria. In the present study, we therefore gated on all HLA DR+ cells whether or not expression of HLA DR was high or low to avoid underestimation of the number of DCs in the peripheral circulation. In agreement with the previous study (33), we show here that the expression of HLA DR was reduced on monocytes and CD1c+ and BDCA3+ mDCs, whereas pDCs showed normal expression of HLA DR. In contrast to the study by Pichyangkul et al., who reported decreased frequencies of pDCs in Thai patients with acute malaria, we did not observe any significant change but a trend toward lower numbers in this DC population (26). The reason for the differences in pDC frequency in these two studies is not clear but could be due to the difference in ethnicity, age, and previous exposure to falciparum malaria, resulting in different kinetics of pDC activation in the two study populations. pDCs are activated by a parasite protein found in schizont lysate or by hemozoin in vitro and show a fundamentally different response to mDCs. In contrast, mDCs are modulated in vitro by the adhesion of iRBC to CD36, as well as by the phagocytosis of hemozoin. In vivo, all of these different mechanisms could act on DC subsets and induce different types of immune response over the course of an infection depending on the dose of the modulating/activating substance, localization of the responding cell type, and the kinetics of the cellular response. Some evidence for such a scenario comes from studies on the DC function in rodent models of malaria. Although some of these studies provide apparently conflicting results, a consensus seems to emerge whereby DC may be activated and induce T-cell responses early during infection but show a modulated phenotype and fail to initiate T-cell responses later during infection (12, 20, 24–27, 29).

We have shown previously in vitro that when monocyte-derived DCs are cocultured with CD36-binding iRBCs they remain phenotypically immature and fail to secrete IL-12, but they do secrete IL-10 and TNF-α. In the present study, we began to investigate whether we could observe a relationship between the cytoadhesion phenotype of iRBC and DC frequency or plasma cytokine concentration during acute disease. We therefore cultured homologous parasites to maturity and analyzed binding to ICAM-1, CD36, CD31, and immunoglobulins in a static binding assay. These assays can only provide results for the average binding of all parasites that have grown to maturity and do not take into account differences in binding between different strains of parasites if a child is infected with more than one strain. Bearing this confounding factor in mind, we report here that the overall concentration of IL-12 in plasma was inversely correlated with the adhesion of iRBCs to CD36, whereas the concentration of IL-10 in plasma showed a positive correlation independent of the effect of parasitemia. Myeloid DCs are the main producers of IL-12, although it is conceivable that monocytes differentiating into DCs after activation via TLRs may be an additional source of IL-12 (17). IL-10 is produced by DCs and monocytes (which express...
the parasites genetic and phenotypic make-up may be able
dressing the interaction between the phenotype and the
icity of the expressed PfEMP-1. Longitudinal studies ad-
serum could then be due to differences in the immunoge-
recognition of a parasite isolate by heterologous immune
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DCs and possibly monocytes, resulting in better T-cell acti-
they induce the production of IL-12 rather than IL-10 by
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immune response to PfEMP-1 and the immune selection of
iRBCs to CD36 and the concentration of IL-10, IL-12, or TNF-α. ICAM-1 is
expressed on lymphocytes including T cells, NK cells, and NKT
plasma concentration of IL-10, IL-12, or TNF-
DC subsets was independent of adhesion to CD36 or the
iRBCs to ICAM-1 and the frequency of CD1c
also observed a positive relationship between the adhesion of
iRBCs to ICAM-1 is direct or indirect, it does
describe further research to establish its biological signifi-
cance.

Previous studies have shown that the binding of iRBCs to
CD36 is higher in children with uncomplicated malaria than in
children with severe disease (22, 28, 30). It is important to
establish whether a relationship between the adhesion of
iRBCs to CD36 and the concentration of IL-10 and IL-12 in
plasma we observed here in children with severe malaria also
exists in children with uncomplicated malaria with the lower
parasitemias commonly found in this group. Furthermore, at
least in the genome of the laboratory line 3D7, a subgroup of
var genes encoding PfEMP-1 has been identified that do not
bind to CD36 (16). Importantly, this subgroup appears to be
predominantly expressed in nonimmune volunteers experi-
entially infected with 3D7 iRBCs during vaccine trials and in
laboratory lines selected with antibodies from children with
severe disease (15, 19). In addition, children with severe malar-
ia and heterozygotic for a null mutation in CD36 are more
likely to be infected with parasites expressing PfEMP-1 with a
high frequency of recognition by heterologous immune se-
rum, indicating that adhesion to CD36 is associated with
parasites with a low frequency of recognition (5). So, what
could be the relationship between adhesion to CD36, the
immune response to PfEMP-1 and the immune selection of
the expressed PfEMP-1 in nonimmune and semi-immune
individuals? One explanation would be that parasites with
no or low binding to CD36 are more immunogenic because
they induce the production of IL-12 rather than IL-10 by
DCs and possibly monocytes, resulting in better T-cell activa-
tion and helper function. A high or low frequency of
recognition of a parasite isolate by heterologous immune
serum could then be due to differences in the immunoge-
necity of the expressed PfEMP-1. Longitudinal studies ad-
ressing the interaction between the phenotype and the
duration of immune responses to P. falciparum infection and
the parasites genetic and phenotypic make-up may be able to
answer these questions.

ACKNOWLEDGMENTS
This article is published with the permission of the Director of the Kenya Medical Research Institute. This study was supported by the Wellcome Trust. B.C.I. holds a Wellcome Trust Career Development Fellowship, and T.N.W. holds a Senior Clinical Fellowship. P.B., C.I.N., and K.M. are supported by the Wellcome Trust. None of the authors reported any conflicting interests.

We thank the children and their parents or guardians for participat-
ing in the study. We also greatly appreciate the help of clinical teams at the KEMRI ward and the Outpatients Clinic and the field workers of the mild malaria study.

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Editor: J. F. Urban, Jr.