Characterization of Peripheral Blood Lymphocyte Subsets in Patients with Acute Plasmodium falciparum and P. vivax Malaria Infections at Wonji Sugar Estate, Ethiopia

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We investigated the absolute counts of CD4+, CD8+, B, NK, and CD3+ cells and total lymphocytes in patients with acute Plasmodium falciparum and Plasmodium vivax malaria. Three-color flow cytometry was used for enumerating the immune cells. After slide smears were stained with 3% Giemsa stain, parasite species were detected using light microscopy. Data were analyzed using STATA and SPSS software. A total of 204 adults of both sexes (age, >15 years) were included in the study. One hundred fifty-eight were acute malaria patients, of whom 79 (50%) were infected with P. falciparum, 76 (48.1%) were infected with P. vivax, and 3 (1.9%) were infected with both malaria parasites. The remaining 46 subjects were healthy controls. The leukocyte count in P. falciparum patients was lower than that in controls (P = 0.015). Absolute counts of CD4+, CD8+, and CD3+ cells and total lymphocytes were decreased very significantly during both P. falciparum (P < 0.0001) and P. vivax (P < 0.0001) infections. However, the NK cell count was an exception in that it was not affected by either P. falciparum or P. vivax malaria. No difference was found in the percentages of CD4, CD8, and CD3 cells in P. falciparum or P. vivax patients compared to controls.

In summary, acute malaria infection causes a depletion of lymphocyte populations in the peripheral blood. Thus, special steps should be taken in dealing with malaria patients, including enumeration of peripheral lymphocyte cells for diagnostic purposes and research on peripheral blood to evaluate the immune status of patients.

In areas of stable malaria endemicity, a heavy burden of morbidity and mortality due to malaria falls on young children, while malaria is a relatively mild condition in adults. This is due mainly to the acquisition of species- and parasitic-stage-specific cellular and humoral immunity against the malaria parasites which increases with age (12, 18, 19).

However, malaria parasites are also known to perturb the normal profile of immune cells in the peripheral blood. For example, total-leukocyte (WBC), total-lymphocyte, NK cell, θα and γδ T-cell, and B-cell counts and T-cell proportions have been reported to be affected by Plasmodium falciparum and Plasmodium vivax infections (15, 31). Although reactive T cells could be detected in a splenic cell population, these cells were not also detectable within the peripheral blood of malaria patients (13). Moreover, no response to antigen stimulation in vitro was observed in peripherally circulating cells in P. falciparum malaria infection (8). A remarkable loss of T cells with high expression of LFA-1 (CD11/CD18) during acute P. falciparum malaria has also been reported by others (4). These findings indicate the withdrawal of lymphocytes from the peripheral blood to body tissue or lymph nodes, where they are sequestered and remain trapped (22). In contrast, others have reported no significance difference in the WBC count, the percentages of CD4+ and CD8+ cells, or the CD4/CD8 ratio in P. falciparum patients (15).

In addition to the induction of sequestration of the immune cells in the lymph nodes, malaria infection is also known to cause apoptosis of the mononuclear cells in humans and animals (6, 16, 24).

Although there is ample evidence showing the potential of malaria infection to affect the counts of lymphocyte subpopulations in the peripheral blood, this might not be consistent in all geographical locations. This is because the pathogenesis as well as the disease outcome of malaria is highly dependent on local factors such as the level of endemicity (26), host genetics (1, 7), and parasite factors (3).

In Ethiopia, although 60% of the population of 70 million is estimated to be at risk of malaria (30), research related to host immunity against malaria is not well established. Therefore, this study aimed to characterize the absolute counts of peripheral blood lymphocyte cells (CD4+, CD8+, CD3+, B, and NK cells and total lymphocytes) in patients with acute P. falciparum or P. vivax malaria.

MATERIALS AND METHODS

Study area and population. This cross-sectional study was undertaken from November 2002 to November 2003 at Wonji Sugar Estate, Ethiopia, 114 km away from the capital city, Addis Ababa. The average elevation of the study area is 1,500 m above sea level, and its climate is characteristic of tropical lowlands. Annual total rainfall is around 8,324 mm, and 65% of the total falls within the months of June to September. Thus, the topography and climatic conditions of the study area are suitable for malaria transmission (17).

Because malaria epidemicity during the study period was very low due to intensive malaria control in the study area, we recruited all adults of both sexes (age, >15 years) acutely infected with P. falciparum or P. vivax who attended Wonji Hospital from November 2002 to November 2003. Informed written consent was obtained from all participants. All malaria cases were treated according to the national standard drug regimens. Clinical and demographic data were recorded by using a standard questionnaire.

The study was undertaken under the auspices of the Ethio-Netherlands AIDS Research Project (ENARP), and its ethics were approved both nationally, by the

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The solution (Becton Dickinson) was added. After vortexing, the mixture was incubated to lyse the red blood cells, 450 μl of the whole blood was transferred to Nunc tubes for FACScan and hematology analyses.

**TABLE 1.** MAbs, fluorochromes, and corresponding peripheral blood lymphocyte populations detected by three-color flow cytometry

<table>
<thead>
<tr>
<th>MAB + fluorochrome(s)</th>
<th>Cell population(s) detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-CD45 + PerCP</td>
<td>All leukocytes</td>
</tr>
<tr>
<td>Anti-CD3 + FITC</td>
<td>All T lymphocytes</td>
</tr>
<tr>
<td>Anti-CD4 + FITC/PE/PerCP</td>
<td>Helper T cells</td>
</tr>
<tr>
<td>Anti-CD8 + FITC/PE/PerCP</td>
<td>Cytotoxic T cells</td>
</tr>
<tr>
<td>Anti-CD19 + FITC/PE/PerCP</td>
<td>B cells</td>
</tr>
<tr>
<td>Anti-CD19/56 + FITC/PE/PerCP</td>
<td>NK cells</td>
</tr>
</tbody>
</table>

* From Becton Dickinson (San Jose, Calif.). PerCP, peridinin chlorophyll protein; FITC, fluorescein isothiocyanate; PE, phycoerythrin.
  a Anti-CD19 plus anti-CD56.

Ethiopian Science and Technology Commission, and institutionally, by the Ethiopian Health and Nutrition Research Institute (EHNRI).

Thus, a total of 204 adults of both sexes were included in the study. One hundred forty-eight subjects had acute malaria infections: 78 (50%) *P. falciparum* malaria patients (median age, 35 years; interquartile range [IQR], 28 to 42 years), of whom 77.2% were males; 76 (48.1%) *P. vivax* patients (median age, 28 years; IQR, 25 to 38 years), of whom 56.7% were males; and 3 (1.9%) patients infected with both *P. falciparum* and *P. vivax* (median age, 29 years; IQR, 24 to 35 years). Forty-six age- and sex-matched healthy adult volunteers (median age, 33 years; IQR, 30 to 39 years) without detectable parasitemia and living in the same area, 84.7% of whom were males, were included as controls. However, eight (4.8%) malaria patients confected with human immunodeficiency virus (HIV) were excluded from the study. There was no age or sex difference among the study participants.

**Sample collection and processing.** Six to eight milliliters of venous blood was collected by venipuncture from each study subject into an EDTA tube. Whole-blood samples, slide smears, and filled-out questionnaires were sent daily to the EHNRI laboratory in Addis Ababa and arrived at about 3 pm. Upon arrival, 600 μl of the whole blood was transferred to Nunc tubes for FACScan and hematological analyses.

**Detection of malaria infections and calculation of parasite densities.** Thick and thin blood films stained with 3% Giemsa stain were examined microscopically. At least 200 microscopic fields were scanned before a smear was regarded as negative. The number of parasites was counted against 300 WBCs. The parasite density per microliter of blood was calculated by multiplying the number of parasites counted by the number of WBCs divided by 300 (25).

**HIV screening.** HIV testing was done using Determine HIV1/2 (Abbott Laboratories, Japan), an enzyme linked immunosorban assay (Vironostika-HIV Uni-Form II Plus; Organon Teknika, The Netherlands), and Western blotting (Genelabs Diagnostics, Singapore) as screening, confirmatory, and tiebreaker assays, respectively.

**Immunophenotyping.** The monoclonal antibodies (MAbs) used for phenotypic characterization of peripheral blood lymphocyte populations are listed in Table 1. To 10 μl of the MAbs in test tubes, 50 μl of whole blood was added and mixed by vortexing. Samples were then incubated for 15 min in the dark at room temperature. To lyse the red blood cells, 450 μl of a fluorescence-activated cell sorter lysing solution (Becton Dickinson) was added. After vortexing, the mixture was incubated for another 15 min at room temperature.

**Flow cytometric analysis.** To obtain absolute counts of lymphocytes, a dual-platform method (using a hematology instrument and a flow cytometer) was applied.

A three-color flow cytometry analysis panel was done using a FACScan flow cytometer (Becton Dickinson). Before data acquisition, instrument parameters were checked and optimized using CaliBRITE beads (Becton Dickinson). Data were acquired with Multiset CellQuest software (Becton Dickinson). For each sample, data for 2,500 lymphocytes were acquired using log-amplified fluorescence and linearly amplified side and forward scatter signals. Data were analyzed with Paint-A-Gate software followed by MultiSET (both from Becton Dickinson). As a control for appropriate lymphocyte gating, the mean percentages of CD4+ and CD8+ T cells were checked to ensure that they fell within a ±10% range of the average percentage of CD3+ cells.

**WBC count.** Absolute counts of WBCs were obtained by using a T540 counter (Coulter Electronics, Florida).

**Statistical analysis.** Data were entered and analyzed using Microsoft Access (DBse IV), STATA (Stata Corporation, Texas), and SPSS (SPSS Inc., Chicago).

**RESULTS**

**Leukocyte counts.** The mean counts of total WBCs were generally lower in patients with both types of malaria, but the difference was significant only for *P. falciparum* patients (*P* = 0.015). Total WBC counts were also lower in *P. falciparum* than in *P. vivax* malaria patients (*P* = 0.031) (Table 2).

**Lymphocyte subpopulation counts.** Almost-twofold decreases in the absolute counts of all CD4+ cells, CD8+ cells, B cells, T cells (CD3+), and total lymphocytes were found in *P. falciparum* patients compared with controls (*P* < 0.0001). Likewise, significant decreases in counts of CD4+ cells, CD8+ cells, B cells, T cells (CD3+), and total lymphocytes were also observed in *P. vivax* malaria patients (*P* < 0.0001). The CD4/CD8 ratio was higher in *P. falciparum* patients (*P* = 0.044) but showed no difference in *P. vivax* infection. The only lymphocyte subset that showed no significant difference in absolute counts for both the *P. falciparum* and *P. vivax* malaria groups compared to healthy controls was NK cells (Table 3).

Compared to the two malaria groups shows that although counts of total lymphocytes and of CD4+, CD8+, NK, B, and CD3+ cells were lower in *P. falciparum* than in *P. vivax* malaria groups compared to healthy controls were NK cells (Table 3).

**TABLE 3.** Absolute counts of total B (CD19+) and NK (CD16 + CD56) cells in *P. falciparum* and *P. vivax* malaria patients and healthy controls

<table>
<thead>
<tr>
<th>Cell type</th>
<th>P. falciparum patients (n = 26)</th>
<th>P. vivax patients (n = 43)</th>
<th>Controls (n = 46)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD19+</td>
<td>61 (±39)ab</td>
<td>86 (±56)ab</td>
<td>192 (±98)</td>
</tr>
<tr>
<td>CD16 + CD56</td>
<td>156 (±157)</td>
<td>180 (±176)</td>
<td>222 (±141)</td>
</tr>
</tbody>
</table>

* a Significant difference when *P. falciparum* or *P. vivax* malaria patients were compared with controls by using the Student *t* test.
  b Significant difference when *P. falciparum* patients were compared with *P. vivax* patients by using the Student *t* test.
patients, the difference was significant only for B cells \( (P = 0.028) \).

**DISCUSSION**

The decrease in total WBC counts during *P. falciparum* malaria \( (P = 0.015) \) was in agreement with earlier reports from other geographical locations \( (20, 31) \). Likewise, the lack of reduction in WBC counts during *P. vivax* malaria was also reported elsewhere \( (31) \). The lower leukocyte counts in *P. falciparum* than in *P. vivax* malaria patients in this study may indicate that immunopathogenesis is more important in the disease due to *P. falciparum* than in *P. vivax* malaria.

A study done by Worku et al. \( (31) \) 10 km from the present study site, which showed significant decreases in CD8\(^+\)-cell, T-cell (CD3\(^+\)), and total-lymphocyte counts in acute *P. falciparum* patients, was in agreement with this study. In contrast, however, those investigators found no change in the absolute counts of CD4\(^+\), B, and NK cells. As in this study, other workers have also reported lower absolute counts of CD4\(^+\), CD8\(^+\), CD3\(^+\), B, and NK cells and total lymphocytes \( (9, 14, 15) \) during acute *P. falciparum* malaria. Although the majority of the studies have shown that malaria infection affects the lymphocyte profiles in peripheral blood, the extent of the decrease and the type of cells altered differ in different geographical locations. This could be due to differences in the immune status of the study subjects related to the level of malaria endemicity \( (26) \), or it could be due to a possible difference in parasite strains, which may cause differences in the activation of the immune system \( (3) \). It could also be due to differences in the baseline values of the absolute counts of the immune cells of the study subjects \( (27) \), or to the impact of geographical locations \( (11) \).

The lack of difference in the absolute counts of NK cells in both *P. falciparum* and *P. vivax* malaria infections in this study has been explained by the rare exit of NK cells from the peripheral blood into lymph nodes or Peyer’s patches, despite their expression (like the other lymphocyte subsets) of several adhesion molecules \( (23) \). This explanation was supported by findings showing that, while significant increases in the numbers of monocytes/macrophages and cytotoxic T lymphocytes were observed in the intravascular space of placentas of acute malaria patients, a complete absence of NK cells was found in all placentas \( (21) \).

In summary, the findings of this study indicate that, although both *P. falciparum* and *P. vivax* infections cause significant decreases in lymphocyte counts, the rate or degree of influence of asexual parasitemia is stronger in *P. falciparum* than in *P. vivax* malaria, a difference that might be related to the level of asexual-stage densities or might be due to antigenic differences between the asexual stages of the two malaria parasites, which might activate the immune system differently.

The findings of this study are very important for countries such as Ethiopia, where 1.5 million people are infected with HIV. Ethiopia has started to implement antiretroviral treatment (ART) \( (29) \). Our findings are related to the eligibility criteria for initiation of ART, which are based on the counting of CD4\(^+\) cells \( (<200/\mu l \text{ of blood}) \) \( (29) \) among other criteria. Therefore, a reduction in the number of CD4\(^+\) cells due to *P. falciparum* or *P. vivax* malaria in patients coinfected with HIV could mislead the physicians to prescribe ART for HIV-positivе individuals who actually should not start antiretroviral drugs. Reductions in the number of CD4\(^+\) cells due to malaria infection could also lead to exaggerated estimates of the total number of HIV-positive people who should start ART in a country where there are overlapping infections with HIV and malaria.

There are probably two main potential mechanisms that could explain the depletion of lymphocyte subsets from the peripheral blood in acute *P. falciparum* and *P. vivax* malaria patients: (i) sequestration of cells into the lymph nodes or other body parts and/or (ii) abnormal death of the cells through apoptosis.

In support of the first hypothesis, sequestration (entrapment of the cells on the lymph nodes and other body organs), several pieces of physiological and immunological evidence have been suggested. The levels of cytokines (tumor necrosis factor alpha and gamma interferon) that are known to induce the expression of the adhesion molecules (selectins, integrins) and chemotactrant chemokines \( (23) \) have been observed to correlate with the severity of malaria caused by *P. falciparum* \( (2, 28) \) and *P. vivax* \( (4) \) infections. Moreover, increases in the levels of these adhesion molecules (ICAM-1 and VCAM-1) in plasma and expression of ECAM-1 on the surfaces of endothelial cells have also been reported during malaria infection \( (4, 10) \). Therefore, the emergence and disappearance of these adhesion molecules during acute malaria infections might prompt different movements of the cells from blood to lymphoid organs \( (5) \), which can result in alterations in the proportions and absolute counts of immune cells in the peripheral blood \( (23) \).

In support of these findings, it has been shown that, while reactive T cells could be detected in a splenic cell population during and after infection, these cells were not detectable within the peripheral blood T cells during acute malaria infection \( (13) \), indicating the withdrawal of T cells away from the peripheral blood to other body tissues.

The second hypothesis that may explain the depletion of the lymphocyte subsets in acute malaria is apoptosis. The occurrence of apoptotic death of the immune cells, which has been shown in studies done with humans and animal models \( (6, 16, 24) \), might support this hypothesis. However, the exact mechanism of apoptotic cell death and its impact on the decrease in the lymphocyte population should be investigated.

In conclusion, our results showed that *P. falciparum* infection causes a significant decrease in total-leukocyte counts. However, both *P. falciparum* and *P. vivax* malaria parasites cause depletion of CD4\(^+\), CD8\(^+\), B, and CD3\(^+\) cells and total lymphocytes but cause no change in NK cell counts. The effect of *P. falciparum* malaria on lymphocyte subset cell counts was greater than that of *P. vivax* malaria, although the difference was not significant.

Based on the findings, we recommend great caution during enumeration of lymphocyte subpopulations in patients infected with *P. falciparum* or *P. vivax* for diagnostic or research purposes. This should also be considered in studies of peripheral blood cells that aim to evaluate the immune status of individuals or to assess immune responses to natural or artificial immunizations, since an optimal number of important cells cannot be obtained. The impact of the depletion of lymphocyte subsets in malaria patients on their susceptibility to coinfection.
with other, new infectious agents and on the clinical consequences of the concomitant infections must be investigated. If apoptosis is contributing to the malaria-associated depletion of lymphocytes, its effect on the parasite and the host should also be evaluated. The possible sequestration of the lost lymphocytes from circulation in the deep capillaries such as the brain and heart, and its effect on the severity of \textit{P. falciparum} and \textit{P. vivax} malaria infections, is another point that must be investigated. The exceptional profile of NK cells, different from those of other lymphocyte subpopulations, also needs investigation.

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