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Rapid Reemergence of T Cells into Peripheral Circulation Following Treatment of Severe and Uncomplicated *Plasmodium falciparum* Malaria

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Frequencies and absolute numbers of peripheral T-cell subsets were monitored closely following acute *Plasmodium falciparum* malaria in 22 Ghanaian children from an area of hyperendemicity for seasonal malaria transmission. The children presented with cerebral or uncomplicated malaria (CM or UM, respectively) or with severe malarial anemia. For all patients the frequencies and absolute numbers of peripheral T cells were lower than normal during the acute stage of disease. This lowering was most pronounced in the CM group and least pronounced in the UM group. Of particular interest, the CM patients showed markedly reduced frequencies of $CD4^+$ cells, the number of which also normalized slower than in the other clinical groups. In all patients, the T-cell frequencies gradually approached normal values after the initiation of therapy, whereas the absolute numbers rapidly reverted from lower than normal to higher than normal before returning to steady-state levels. Furthermore, the initially reduced T-cell surface density of the T-cell receptor/CD3 complex, which rapidly normalized, was a general finding for all three clinical groups, suggesting a state of peripheral T-cell hyporesponsiveness during acute malaria. The data presented suggest a rapid therapy-induced reemergence of T cells that had been temporarily removed from the peripheral circulation as a consequence of the malaria attack and that the degree of the disease-induced T-cell reallocation correlates with disease severity.

Acute *Plasmodium falciparum* malaria is associated with marked perturbations of the cellular immune system (reviewed in reference 8). Changes include markedly lowered frequencies and absolute numbers of T cells in the peripheral circulation and a transient inability of such cells to respond to antigenic stimulation in vitro. These findings appear to reflect a temporary reallocation of T cells away from the periphery in response to the infection (2, 9). Few studies have addressed the kinetics of this redistribution event (1, 19), and none have examined these changes as they occur in children from areas where malaria is endemic, who bear the burden of malaria-related morbidity and mortality. Furthermore, no studies have systematically examined putative differences in these perturbations in immunity with respect to the clinical presentation of the disease, from uncomplicated malaria to life-threatening severe malarial anemia and cerebral malaria.

Here we report the results of a detailed investigation of peripheral T-cell frequencies and absolute numbers in children from an area of hyperendemicity for seasonal malaria transmission in Ghana following episodes of acute *P. falciparum* malaria, presenting as cerebral malaria (CM), uncomplicated malaria (UM), or severe malarial anemia (SA).

MATERIALS AND METHODS

Donors. A total of 22 donors were included in the study. All donors were pediatric patients (ages, ≥3 years) admitted to the Department of Child Health, Korle-Bu Teaching Hospital, Accra, Ghana, with a diagnosis of CM ($n = 9$), UM ($n = 8$), or SA ($n = 5$). All donors had asexual *P. falciparum* parasitemia and were febrile (≥37.5°C) at admission. Diagnostic criteria for the CM group consisted of a Blantyre coma score of <3 (16) lasting for ≥30 min and not responding to intravenous glucose and exclusion of meningitis, encephalitis, head trauma, diabetes, and history of neurological disorder. For the UM group, the criteria were full consciousness, asexual parasitemia of ≥10,000 parasites/μl, hemoglobin of ≥8 g/dl, and exclusion of other febrile disease. Finally, only fully conscious patients with asexual parasitemia of ≥10,000 parasites/μl and hemoglobin of ≤5 g/dl in the absence of recent severe bleeding or other known cause of anemia were included in the SA group. Apart from coma scores and hemoglobin levels, clinical characteristics such as age, axillary temperature, symptom duration prior to admission, and parasitemia were similar in the three clinical groups (14).

All children were treated with a standard regimen of chloroquine, which is still effective against severe malaria in Accra, with a case fatality rate for strictly defined CM of approximately 6% at the Department of Child Health, Korle-Bu Teaching Hospital (13a, 17), and all recovered fully. Patients with hemoglobin S disorders were excluded from the study.

Seven healthy age-matched donors from a nearby community were included as controls (CC donors).

The study was approved by the Ethical and Protocol Review Committee, University of Ghana Medical School.

Blood samples and flow cytometry. Samples of heparinized, peripheral blood (250 to 400 μl) were obtained at admission before the initiation of therapy (day 0) and subsequently on days 1, 2, 3, 7, 14, and 30. Samples were obtained only after informed consent was given by parents or guardians.

Following automated hematological analysis (Haemat 12; SEAC, Firenze, Italy), the samples were centrifuged, and the plasma was removed. After resuspension in 400 μl of phosphate-buffered saline, 50-μl aliquots were labelled (20 min, room temperature) with combinations of directly fluorochrome-conjugated monoclonal antibodies to CD3 (clone SK7; Becton Dickinson, San Jose, Calif.), CD4 (clone SK3; Becton Dickinson), or CD8 (clone SK1; Becton Dickinson) and CD11a (LFA-1; clone MHM24; Dako, Glostrup, Denmark) or with nonspecific isotype control antibodies. Erythrocytes were subsequently lysed with fluorescence-activated cell sorter lysing solution (Becton Dickinson), washed twice in phosphate-buffered saline–3% fetal calf serum, and analyzed on a FACScan flow cytometer (Becton Dickinson). Samples were live gated on lymphocytes by forward and side scatter, and data for 10,000 events were collected.

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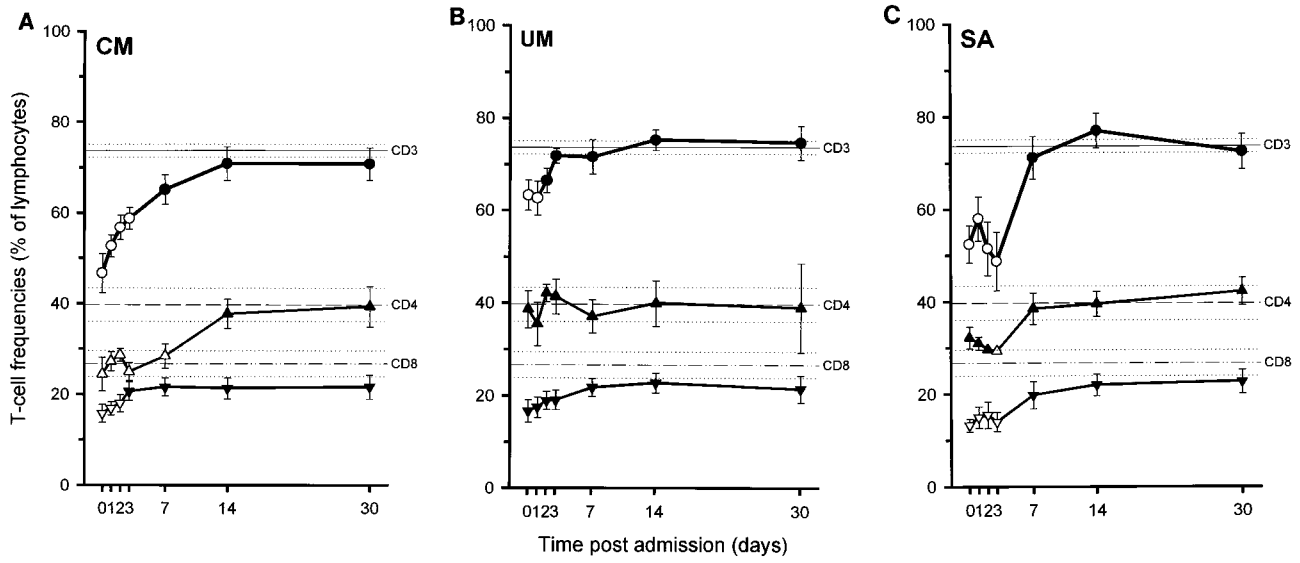

FIG. 1. Frequencies of peripheral $CD3^+$ (circles), $CD4^+$ (triangles), and $CD8^+$ (inverted triangles) T cells following admission (day 0) of patients with acute *P. falciparum* malaria: CM (A), UM (B), or SA (C). Datum points are the mean percentages of lymphocytes ± SEM (error bars). Mean frequencies (dashed lines) ± SEM (dotted lines) of the corresponding subsets in a group of healthy age-matched children from a nearby community are shown for comparison. Mean frequencies of a subset for the CM, UM, and SA groups that were significantly different ($P < 0.05$) from the corresponding values for the CC group are indicated by open symbols.

Data presentation and statistical analyses. Data are presented as means ± 1 standard errors of the means (SEM). Complete sets of datum points were not available for all donors, and time points for which fewer than four individual datum points within a clinical group were available were excluded. Average cell frequencies and numbers at different time points were compared to the corresponding values for a control group of age-matched, healthy children by one-way analysis of variance, supplemented by Dunnett's test. Comparison between clinical groups at different time points was done by one-way analysis of variance supplemented by Student-Newman-Keul's test. Correlation analysis was done by Pearson product-moment correlation. SigmaStat software (Jandel Scientific, San Rafael, Calif.) was used for the statistical analyses.

RESULTS

Frequencies of $CD3^+$, $CD4^+$, and $CD8^+$ T cells. The results of analyses of T-cell population frequencies at different time points in the three clinical categories are summarized in Fig. 1. All patient groups presented (day 0) with reduced T-cell frequencies, which gradually normalized toward values obtained from a panel of age-matched, healthy control donors (CC). Frequencies of $CD3^+$, $CD4^+$, and $CD8^+$ cells were significantly different ($P < 0.05$) from the corresponding values for the CC donors (Fig. 1).

Normalization of T-cell frequencies was slowest in the CM group, which also showed the most-pronounced perturbations, and most rapid in the UM group, in which the effect of the disease episode had the least impact on T-cell frequencies. In the SA group, the normalization was consistently interrupted between days 1 and 3, coinciding with the time of blood transfusions given to these patients, rendering interpretation of differences in results between this and the other groups difficult at these time points. Interestingly, CD4 frequencies were markedly reduced in the CM patients, were reduced less in the SA group, and apparently were virtually unaffected in the UM group (CD4 frequencies in CM and UM patients were significantly different [$P < 0.05$] on days 0 and 2 to 7). In contrast, CD8 frequencies were similar in the different clinical groups at all time points ($P > 0.6$).

Absolute numbers of $CD3^+$, $CD4^+$, and $CD8^+$ T cells. The absolute numbers of T cells were almost halved at admission (Fig. 2) due to both lymphopenia and the reduced T-cell frequencies within the lymphocyte population. However, in contrast to the gradual normalization of T-cell frequencies, the initially very low T-cell counts rapidly increased to numbers clearly above normal around day 7, at which average counts were 25 to 50% above normal, and then returned to steady-state levels only around day 14. The overall patterns of the numbers of $CD3^+$ and $CD8^+$ T-cells at various time points were similar between the different clinical groups ($P > 0.23$, except on day 3 when the results for the CM and SA groups were significantly different, probably due to the impact of transfusions in the latter group [see previous section]). In contrast, while the absolute numbers of $CD4^+$ cells never increased much above normal in the CM group, they did increase in the other two groups (difference between CM and UM results being statistically significant on day 3 [$P < 0.05$]).

CD3 fluorescence. In addition to reduced frequencies and absolute numbers of $CD3^+$ cells, T cells from all patients examined were initially characterized by reduced geometric mean CD3 fluorescence, which rapidly increased toward normal levels within the first few days of therapy. The data for the CM patients are shown in Fig. 3, but similar results for the other clinical groups were obtained. Although there was a tendency toward lowered FSC signals (a surrogate marker of cell size) within the $CD3^+$ cells during early time points, there was no significant correlation between the FSC and CD3 signals (P [$r = 0.12$] = 0.19; $n = 126$). Thus, it is unlikely that the initial reduction in CD3 fluorescence merely reflects reduced cell size, but rather it suggests that the average surface density of CD3 molecules on peripheral T cells obtained at or shortly after admission was reduced compared to that for healthy donors. This reduction in CD3 fluorescence was observed within both the $CD4^+$ and the $CD8^+$ subsets (data not shown).

T cells with high LFA-1 expression. Both the frequencies and the absolute numbers of $CD3^+$, $CD4^+$, and $CD8^+$ cells characterized by high surface density of the T-cell adhesion molecule LFA-1 were initially lower than normal (data not shown). Frequencies generally approached normal within 1 week, whereas the initially low absolute numbers were above

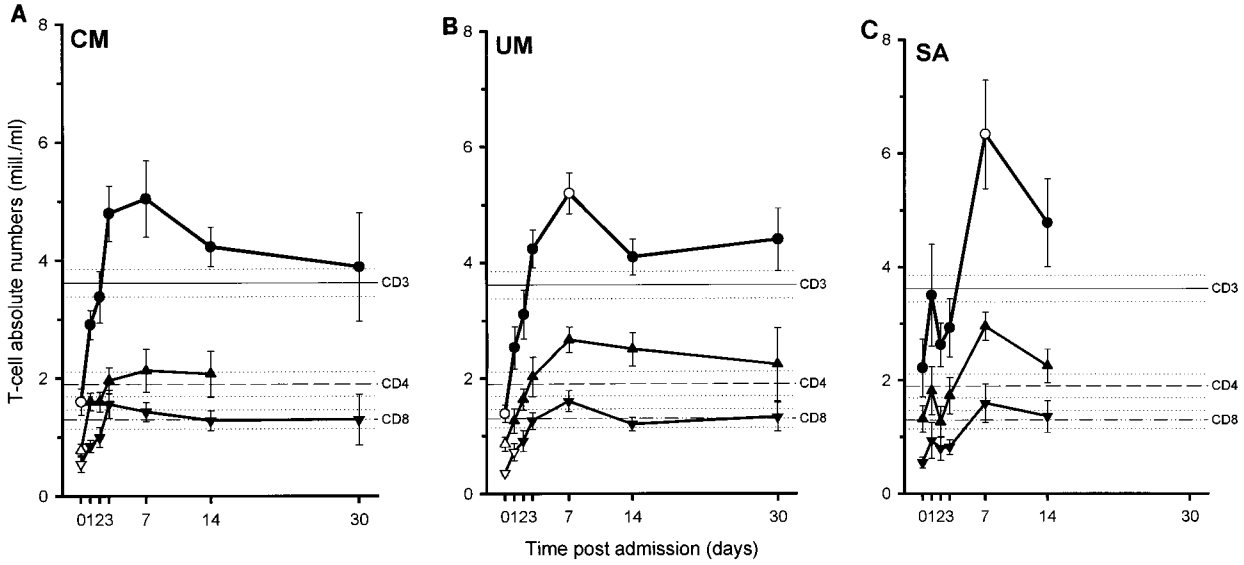

FIG. 2. Absolute numbers of peripheral $CD3^+$ (circles), $CD4^+$ (triangles), and $CD8^+$ (inverted triangles) T cells following admission (day 0) of patients with acute *P. falciparum* malaria: CM (A), UM (B), or SA (C). Data presentation is described in the legend to Fig. 1. Mill., 10^6.

normal at this time and returned to normal around day 14. All three clinical groups were similar in this respect.

DISCUSSION

Lymphopenia with reduced frequencies and absolute numbers of T cells in the peripheral circulation are well-known characteristics of patients with acute *P. falciparum* malaria, including children from the areas of Africa where malaria is endemic (5, 22). Although the cause of the phenomenon is unclear, a temporary disease-induced reallocation of T cells has long been regarded a likely explanation (21, 22) and is supported by more-recent studies of human and experimental murine malaria infections (9, 15). Nevertheless, little is known about the duration of this lymphopenia, as only two detailed longitudinal studies, both of adult patients with little or no previous exposure to malaria, have been done (1, 19). The latter of these monitored two patients after experimental sporozoite-induced infections, which were cleared while parasitemias were very low (<300/μl), and thus may not be directly comparable to studies of naturally acquired infections. No studies have examined the relationship between changes in the above-described parameters and clinical presentation in well-defined groups of patients or in the individuals who bear the burden of malaria-related morbidity and mortality, i.e., children in sub-Saharan Africa.

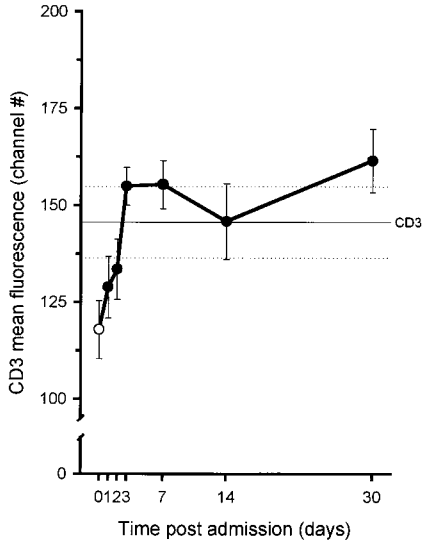

FIG. 3. CD3 fluorescence following admission (day 0) of patients with CM. Data presentation is described in the legends to Fig. 1.

As previously reported, we found lowered frequencies and absolute numbers of all investigated T-cell populations, the degree of which correlated with disease severity, i.e., more pronounced in SA patients and particularly in CM patients than in UM patients. While interpretation of postadmission data from the SA group is complicated by the necessary transfusions of these patients, this observation contrasts with the results of a previous study (1), which reported the absence of such a relationship but no clear definition of severity. Furthermore, that study was concerned with a very different donor population, i.e., adults, predominantly of Caucasian descent, with little or no previous exposure to malaria. Nevertheless, in both studies it is evident that the initially reduced numbers of T cells rapidly changed to numbers above normal before leveling off. Supposing that the reallocation hypothesis is true, an idea supported by the selective depletion of T cells with high surface expression of the important T-cell adhesion molecule LFA-1 in acute *P. falciparum* malaria (references 2 and 9 and this study), this passing increase in the number of peripheral T-cell likely reflects a rapid reemergence of cells sequestered as a consequence of the malaria episode, perhaps supplemented by daughter cells resulting from proliferation of such activated T cells outside the peripheral circulation. Furthermore, there is ample evidence that systemic T-cell activation is indeed a feature of acute malaria, as evidenced by increased levels of soluble markers of T-cell activation in plasma or serum, e.g., soluble interleukin-2 receptor, soluble CD4, and soluble CD8 (2, 10, 12, 13), and that the degree of this activation correlates with disease severity (11, 18). For future investigations of the roles of human T cells in the protection against

and the pathogenesis of malaria, the most useful and accessible cell populations to study may be those obtainable from recovering patients, in whom the numbers and frequencies of relevant T-cell specificities can reasonably be expected to be optimal.

Another interesting observation emerging from the present study is the particularly depressed frequencies of $CD4^+$ cells in the CM patients compared to those in the other clinical groups. Although great care should be exerted in comparing human and experimental murine CM, it is tempting to speculate that this observation reflects pathological recruitment of $CD4^+$ cells into inflammatory sites in CM patients, as $CD4^+$ cells are critically involved in the pathogenesis of experimental CM (4). However, additional studies that explore this possibility are clearly needed.

Peripheral T cells from acutely ill malaria patients have been shown to be unresponsive to antigenic stimulation in vitro (7, 10). Assuming reallocation, the consequent reduction in the frequency of potentially antigen-reactive T cells in the peripheral circulation may be sufficient to explain this in vitro nonresponsiveness. In any case, little evidence supports the alternative hypothesis that some immunosuppressive mechanism operates in vitro, as suppressive $CD8^+$ cells from acute malaria patients are functionally deficient and as removal of putative suppressive cell populations are ineffective in restoring the in vitro T-cell responses (3, 6). However, our finding of reduced surface density of CD3 molecules on circulating T cells from acutely ill patients may indicate that T cells present in the peripheral circulation during acute disease are nevertheless functionally compromised. Down-regulation of the T-cell receptor (TcR)/CD3 complex is a physiological consequence of antigenic stimulation and results in a temporary hyporesponsiveness to subsequent stimulation, since T-cell activation requires engagement of a certain minimum number of TcR/CD3 complexes (20).

In summary, we present evidence suggesting a rapid reemergence of previously sequestered T cells following drug cure of *P. falciparum* malaria patients, that the degree of the disease-induced T-cell reallocation correlates with disease severity, and that T cells present in the peripheral circulation during acute malaria are functionally compromised due to down-regulation of the TcR/CD3 complex.

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