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Cytokines and Malaria Parasitemia

Janine Jason,* Lennox K. Archibald,† Okey C. Nwanyanwu,‡ Michael Bell,† Ian Buchanan,* Joshua Larned,* Peter N. Kazembe,§ Hamish Dobbie,§ Bharat Parekh,* Martha G. Byrd,* Angelia Eick,* Alison Han,* and William R. Jarvis†

*HIV Immunology and Diagnostics Branch, Division of AIDS, STD, and TB Laboratory Research; †Investigation and Prevention Branch, Hospital Infections Program, National Center for Infectious Diseases; and ‡Office of Global Health, Centers for Disease Control and Prevention, U.S. Department of Health and Human Services, U.S. Public Health Service, Atlanta, Georgia 30333; and §Lilongwe Central Hospital, Ministry of Health and Population and Community Health Sciences Unit, Ministry of Health and Population, Lilongwe, Malawi, Africa

The balance between pro- and antiinflammatory cytokines may be important in malaria presentation and outcome. Malaria tends to be more severe in children than in adults, presumably because partial immunity develops with age. However, the full nature of, and age-related differences in, anti-malarial immunity are unknown. We compared: (1) serum and cell-specific cytokines of patients with acute malaria to those of patients with other acute illnesses and to those of healthy adults and (2) the cytokine responses of parasitemic children and parasitemic adults. Flow cytometry was done on the peripheral blood mononuclear cells of 148 hospitalized children, 161 febrile hospitalized adults, and 20 healthy adults in Malawi, Africa, a malaria-endemic country. Serum cytokines were also assessed for 80 of these patients. Thirty-eight participants were parasitemic with *Plasmodium falciparum*. Serum interleukin (IL)-10 (an antiinflammatory, immunoregulatory, and type 2 cytokine) levels were higher in malaria patients than in other patients (medians 502 pg/mL vs 16 pg/mL, $P = 0.002$), and the percentages of various lymphocyte populations making IL-6 (a proinflammatory, type 2 cytokine regulating iron distribution) were lower in malaria patients than in other patients (e.g., for spontaneous production by children's $CD8^+$ T cells: medians 1.4% vs 33.1%, $P = 0.004$). For adult patients, the percentages of lymphocytes spontaneously making IL-4 (a type 2 cytokine) were significantly lower in those with malaria than in those without malaria (medians 0.9% vs 2.1%, $P = 0.005$). The percentages of monocytes spontaneously making IL-8 (a chemotactic, proinflammatory chemokine) were higher in parasitemic children than in parsitemic adults (medians 5.8% vs 1.7%, $P = 0.003$). A number of cellular proinflammatory, type 1 parameters were significantly higher in all children (with or without malaria) than in all adults; these included the percentages of various lymphocyte populations making IL-6, both IL-6 and interferon-γ, or IL-8. These data support the importance of IL-10 in malaria parasitemia. Given the lack of an IL-4 (type 2) response, IL-10's primary role may be immunoregulatory rather than type 2 in nature. In this study, the immune response to malaria was more proinflammatory in children than in adults. This difference, if corroborated by other studies, could be related to malaria's greater severity in children.

Key Words: **malaria; HIV; cytokines; IL-6; IL-8, IL-10.**

INTRODUCTION

Malaria is a major cause of morbidity and mortality in tropical countries. In areas where malaria is endemic, most deaths occur in children. With age, partial immunity develops, leading to partial parasite clearance and less symptomatic or asymptomatic parasitemia. Antiparasite antibodies are one factor in this partial immunity (1). However, an increasing body of evidence from both murine models and human studies indicates that T cells, monocytes, and cytokines may be important in determining the level of malaria parasitemia and rates of clearance (2, 3). Specific cytokines reported to be of importance in human malaria include tumor necrosis factor-α (TNF-α) (4, 5), interferon-γ (IFN-γ) (6–8), interleukin (IL)-6 (4, 7), and IL-10 (3–7, 9).

Some studies suggest that the balance between type 1 and type 2 immune responses, and that between pro- and antiinflammatory responses, determines the degree of malaria parasitemia, level of anemia, clinical severity, and presentation and/or outcome. Murine models suggest that parasite clearance requires an early IFN-γ (type 1) response, followed by an IL-10 (type 2, but also immunoregulatory) response, needed to complete parasite removal (2). Ho *et al.* showed that parasite clearance was related to levels of both IL-10 and IFN-γ but severe malaria was associated with an intense type 1 response (5). Other studies support the idea that the absolute levels of both type 1 and type 2 cytokines determine disease outcome. Extreme levels

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of either pro- or antiinflammatory cytokines may be associated with disease severity and mortality. Day *et al.* found mortality to be associated with high levels of IL-6, IL-10, and TNF-α, the last of these being a strong proinflammatory, type 1 cytokine (4). Similarly, in nonmalarial sepsis, both serum IL-10 and TNF-α were highest in those who died (10).

We had a unique opportunity to explore the roles of type 1 and type 2 cytokines in malaria parasitemia by assessing cellular and serum immune parameters and peripheral blood malaria films of hospitalized adults and children in Malawi, Africa, where malaria is endemic. Cellular immune studies and malaria smears were also done on 20 healthy adult volunteers, 2 of whom were parasitemic. We did not assess the relationships between cytokines and clinical presentation and outcome. Rather, we assessed the relationships between cytokines and the presence or absence, and degree, of parasitemia, a relatively objective parameter. Further, in addition to comparing parasitemic patients to healthy individuals, we compared them to other acutely ill, hospitalized patients, who might be expected also to have alterations in their cytokine profiles. Thus, we could better differentiate parameters specifically associated with malaria parasitemia from those related to severe illnesses in general. We examined four issues: (1) the relationships between immune parameters (including pro-/antiinflammatory cytokines and type 1/type 2 cytokines) and the presence or absence of malaria parasitemia, (2) the relationship between cellular or serum cytokine levels and the degree of malaria parasitemia, (3) differences between children's and adults' cytokine responses to malaria that might be related to the more severe presentation in children, and, since HIV is also endemic in Malawi, (4) the relationships between malaria parasitemia and HIV infection or severity of infection.

PARTICIPANTS AND METHODS

Patients

During three periods in 1997 and 1998, we enrolled all 479 febrile (oral temperature $>$ 38°C) adults ($\geq$13 years old) and all 244 children ($<$13 years old) admitted to the Lilongwe Central Hospital, Malawi, Africa, into a study of bloodstream infections. We enrolled a random subset of these into a substudy of the immune correlates of infectious diseases (148 children and 161 adults, Table 1), as well as enrolling 20 healthy individuals ("controls"). All children admitted to the hospital during the enrollment period were included in the study, irrespective of their temperature at admission, since infected children do not necessarily present with fever. Selection for the substudy was random; participants were comparable to the rest of the study group. The 20 controls were all healthy, actively working adults; 14 were HIV^+, 13 of whom had plasma HIV viral RNA levels measured. Children were not included in the control group for ethical reasons. For each patient, epidemiologic data and a medical history were obtained and a physical examination was performed by one of the investigators. All patients had cellular immune and intracellular cytokine testing done at admission; 65 adult and 15 pediatric patients with sufficient amounts of stored sera also had serum cytokines levels assessed. Epidemiologic data and serum cytokine measurements were not obtained for control participants. The study protocol was approved by the institutional review boards of the Centers for Disease Control and Prevention (CDC) and the Malawian Health Sciences Research Committee; informed consent was obtained from all participants or their guardians.

Laboratory procedures

(i) Malaria. Thick and thin smears were done at admission and read by a single, highly experienced individual not aware of the patient's clinical status or findings. A smear was considered positive if any *Plasmodium falciparum* asexual parasites were seen on examination of peripheral blood smears (thick films and the tails of thin films). The level of parasitemia was graded from 1 to 4, with increasing levels of parasite density as follows: 1+ = $>$0 to 1 parasite/100 red blood cells (RBC), 2+ = $>$1–2/100 RBC, 3+ = $>$2–3/100 RBC, and 4+ = $>$3/100 RBC, a scale that is frequently used clinically and in other published studies (11–13). These levels were used as indicators of severity of parasitemia/immunologic disease control. All patients with positive smears were treated with chloroquine, quinine, or Fansidar.

(ii) HIV. HIV antibody testing was done at study enrollment. As in most developing nations, HIV-infected persons in this study were not receiving antiviral therapies, nor were they being monitored for changes in CD4 counts or HIV viral RNA levels. However, for 47 adult patients, 37 children, and 13 controls, plasma HIV-1 viral RNA levels were assessed at CDC with Roche Monitor version 1.5 test kits (Roche Diagnostics, Indianapolis, IN),[1] which have a lower detection limit of 400 copies/mL.

(iii) Blood cultures. Blood cultures were performed as described previously (14). Bactec Myco/F Lytic bottles (Becton–Dickinson Microbiology Systems, Cockeysville, MD) were incubated at 35°C for 7 days and examined each day. These culture techniques readily detect pathogenic bacteria, fungi, and mycobacteria

[1] Use of all trade names and commercial sources is for identification only and does not imply endorsement by the Public Health Service or the U.S. Department of Health and Human Services.

species (14). Further mycobacterial studies were done in the microbiology laboratory of Duke University Medical Center. For the bacteremic persons discussed herein, organisms included Gram-positive cocci (29% of isolates), Gram-negative rods (51%), and mycobacteria (20%).

(iv) Cytokine stimulation. Blood was prepared for cytokine stimulation as described previously (14). Blood was either stimulated for 5 h at 37°C with phorbol 12-myristate 13-acetate (PMA) (200 ng/mL) (Sigma Chemical Co., St. Louis, MO) and ionomycin (4 μg/mL) (Sigma) in the presence of brefeldin-A (40 μg/mL) (Sigma) and RPMI 1640 with L-glutamine (induced cytokine expression) or retained in identical medium without PMA and ionomycin but with brefeldin-A (spontaneous cytokine expression). No serum was added to the cultures. After being washed, the red blood cells were lysed with ammonium chloride solution and lymphocytes were permeabilized and fixed using Ortho Permeafix (Ortho Diagnostic Systems, Inc., Raritan, NJ). After processing, samples were shipped at 4–8°C to CDC for further analysis. In analyses, the percentage of lymphocytes expressing CD4 was based on data for unstimulated cells.

(v) Flow cytometric reagents. The surface antigens assessed in this study were ones shown in our laboratory to be stable with this permeabilization/fixation protocol (data not shown), i.e., using these techniques, we had comparable results for the surface-related antigens when staining was done either pre- or postpermeabilization. Fluorescein isothiocyanate (FITC)-conjugated, phycoerythrin (PE)-conjugated, peridinin chlorophyll protein (PerCP)-conjugated, or allophycocyanin (APC)-conjugated murine monoclonal antibodies were obtained from the following sources: (a) Becton–Dickinson Immunocytometry Systems/PharMingen (BD/PMG; San Jose, CA) (CD8–FITC and –PE [clone SK1], CD3–PerCP and –APC [clone SK7], CD4–APC [clone SK3], CD45–FITC [clone 2D1], CD19–APC [clone SJ25C1], CD14–PE [clone MϕP9], CD69 [clone L78], CD16–PE [clone B73.1], and CD56 [clone MY31], IL-4–PE [clone 8D4-8], IL-8–PE [clone G265-8], and IL-10–PE [clone JES3-9D7], (b) Research and Diagnostics (Minneapolis, MN) (IL-6–PE [clone 1927.311]), (c) Immune Source (Reno, NV) (CD8–APC [clone KL.12], IL-2–APC [R-56.2], TNF-α–FITC [clone DTX.34], and IFN-γ–APC [clone 13.TR]), and (d) Sigma *(microtubulin [clone DM1A])* custom conjugated to FITC by CalTag, South San Francisco, CA). Isotype controls were obtained from BD/PMG.

(vi) Flow cytometry. All staining was done at room temperature for 30 min in the dark after permeabilization, fixation, and shipment to CDC. Staining was followed by a buffered saline wash. Four-color cytofluorometry was done using a FACSort or FACSCalibur flow cytometer and CellQuest software (BD/PMG). Between 50,000 and 80,000 ungated events were collected from each tube in the panel. Lymphocytes were defined on the basis of forward and side scatter and monocytes on a wide gate based on forward and side scatter of stimulated and unstimulated $CD14^+$ cells.

(vii) Serum cytokines. Serum samples were analyzed for IL-2, IL-4, IL-6, IL-8, IL-10, IFN-γ, and TNF-α by enzyme-linked immunosorbent assays using pairs of cytokine-specific monoclonal antibodies according to the manufacturer's instructions (BD/PMG). Each plate included a standard curve of recombinant human cytokine and known positive and negative controls. All specimens were measured in duplicate, and the means of the two values were used in all analyses. For samples with nondetectable levels, the level for analysis was set at half the detection limit for that assay (detection limit for IL-2, IL-8, IL-10, IFN-γ, and TNF-α, 7.8 pg/mL; for IL-6, 4.7 pg/mL; and for IL-4, 15.6 pg/mL). We did not have IRB approval for drawing nonheparinized blood from control participants; therefore, their serum cytokine levels were not examined. Serum IL-10 levels are usually very low or nondetectable in healthy persons (10, 15–18).

Analytic Techniques

For each participant, analyses were done for all lymphocytes, $CD3^+$ lymphocytes, $CD3^+CD8^+$ lymphocytes, $CD3^+CD8^-$ lymphocytes, $CD3^+CD16/56^+$ lymphocytes, $CD3^-CD16/56^+$ lymphocytes, $CD19^+$ (B) lymphocytes, and/or monocytes. HIV disease severity was based on the percentage of unstimulated cells expressing CD4 and the HIV plasma viral titer. The degree of parasitemia was based on the 1+ to 4+ scale described above.

Statistical Techniques

For relevant analyses, participants were dichotomized according to the presence or absence of parasitemia (smear + vs −), the degree of parasitemia (1+ and 2+ vs 3+ and 4+), and/or age ($<$13 years old vs $\geq$13 years old). Comparisons were made between malaria-positive/blood-culture-negative patients and malaria-negative/blood-culture-negative patients, malaria-negative/blood-culture-positive patients, and malaria-negative controls. Proportions were compared using Fisher's exact or χ^2 tests. Nonparametric comparisons were done using Wilcoxon rank sum and Kruskal–Wallis tests. Spearman's rank correlations (r_s) were computed to assess correlations between immune parameters and levels of parasitemia and between various continuous variables. For HIV-positive

persons having undetectable viral RNA levels, the analysis value was set at half the lower limit of the assay (200 copies/mL). For logistic regression analyses (LRA), statistics provided herein are for the final, reduced model containing only the significant variables. Initial models included body temperature as an independent variable. The significance level for all analyses was set at $P < 0.03$; data not provided herein did not reach that level of significance on any type of analyses.

RESULTS

Participant Characteristics

Of the 148 pediatric and 161 adult patients, 24 adults and 12 children had positive smears for *P. falciparum* and were blood-culture-negative; two control participants had positive *P. falciparum* smears. Three additional patients had positive malaria smears and were blood-culture-positive; these were excluded from all analyses. Parasitemic children did not differ from other blood-culture-negative or blood-culture-positive children in gender, age, mortality, or HIV status (Table 1). Parasitemic adults did not differ from other blood-culture-negative participants, but were younger than the blood-culture-positive participants ($P = 0.002$), had lower mortality ($P = 0.021$), and had lower HIV seropositivity ($P < 0.001$).

Immune Parameters and Presence of Parasitemia

For both adults and children, serum IL-10 levels were an order of magnitude higher in patients with malaria parasitemia (medians 632 pg/mL for adults with malaria, $n = 18$, and 333 pg/mL for children with malaria, $n = 6$) than in patients without parasitemia (medians 16 pg/mL for adults without malaria, $n = 46$, and 23 pg/mL for children without malaria, $n = 9$) (Fig. 1). For children, the median percentage of $CD3^+$(T) lymphocytes and the median percentage of $CD3^+CD8^+$ lymphocytes spontaneously making IL-6 were significantly lower in those with malaria compared to hospitalized, blood-culture-negative children and blood-culture-positive children (Table 2). Similarly, parasitemic adults had a significantly lower median percentage of lymphocytes making induced IL-6 than did the other two patient groups (Table 2). However, the parasitemic adults had a higher percentage of lymphocytes making induced IL-6 than did nonparasitemic controls (0.1%, $P = 0.026$). The percentage of lymphocytes spontaneously making IL-4 was also significantly lower in parasitemic adults than in other patients but was higher than in control adults (0.3%, $P = 0.003$). The only parameter for which the parasitemic group was higher than the other hospitalized groups, and thus was specific for malaria parasitemia, was serum IL-10. None of the parameters above were significantly correlated with patients' temperatures (data not shown, P's associated with r_s's all >0.05), nor was patient temperature a significant independent variable when put into LRA, with each of the variables in Table 2 as a second independent variable and malaria-smear-positivity as the dependent variable (data not shown).

Children vs Adults

Rate of parasitemia did not vary significantly by age, nor did it differ significantly among or between pediatric patients, adult patients, or adult controls (8, 15, and 10%, respectively). The degree of parasitemia also did not vary significantly by age or by age group (data not shown). Although malarial adults had a higher median level of serum IL-10 level than did malarial children, variability was high and serum IL-10 levels did not differ significantly between malaria-positive adults and children. The range in percentage and median percentage of monocytes spontaneously producing IL-8 was greater for pediatric malaria patients than for adult malaria patients, for whom the percentage of monocytes spontaneously producing IL-8 was quite low (medians 5.8%, $n = 12$, vs 1.9%, $n = 26$, $P = 0.003$) (Fig. 2). This remained significant in a LRA ($b = 0.151$, $P = 0.004$). (Results were also significant without the inclusion of the two malaria-positive controls; data above include these two controls.) This age group difference was not present for participants without malaria. For nonmalaria patients, most individuals had low percentages of monocytes spontaneously producing IL-8, but a scattering of both adults and children had relatively high percentages (Fig. 2). A number of other cellular proinflammatory cytokine parameters were significantly higher in children than in adults; these age differences were present in both the malaria-smear-positive and the smear-negative groups. Results for smear-positive patients are provided in Table 3.

Degree of Parasitemia

Among participants with positive malaria films, degrees of parasitemia were measured for 20 adult patients, 2 controls, and 12 children. Serum IL-10 levels had no clear or statistically significant association with level of parasitemia, being highest at level 3+ and lowest at level 4+ (medians: 1+, 502 pg/mL; 2+, 610 pg/mL; 3+, 643 pg/mL; and 4+, 263 pg/mL). For parasitemic children, the percent of monocytes spontaneously making IL-8 declined significantly with increasing parasitemia, with levels comparable to those of adults at level 4+ ($r_s = -0.73$, $P = 0.007$; $b =$

TABLE 1

Characteristics of Patient Participants, by Age Group and Malaria/Blood Culture (BC) Status[a]

	Children (<13 years old)				Adults (≥13 years old)			
	Malaria$^+$/BC$^-$ (n = 12)	Malaria$^-$/BC$^-$ (n = 115)	Malaria$^-$/BC$^+$ (n = 21)	P value[b]	Malaria$^+$/BC$^-$ (n = 24)	Malaria$^-$/BC$^-$ (n = 96)	Malaria$^-$/BC$^+$ (n = 41)	P value
Gender (% male)	67	49	70	*NS*	38	46	54	*NS*
Age in years								
Mean	2.9	2.9	2.6		28	29	36	
Median	3.3	2.0	1.7	*NS*	26	28	35	<0.001
Range	0.2–7	0.1–12	0.1–12		14–50	13–60	21–61	
Mortality rate (%)	0	5	0	*NS*	6	13	35	<0.001
% HIV-antibody-positive	17	25	35	*NS*	52	72	85	0.022

[a] Data incomplete for various individuals. Three patients with both positive smears and positive blood cultures were excluded from the above table and all analyses. Gender not recorded for 1 child and 5 adults; age not recorded for 1 child and 7 adults. HIV status unknown for 3 children and 2 adults; clinical outcome unknown for 49 children and 23 adults. CD4 data technically unacceptable for 1 child and 3 adults. Twenty healthy adult control participants also had malaria smears done, of whom 2 were smear positive and 14 were HIV$^+$; demographic data were not obtained for these individuals.

[b] χ^2 test used for gender and HIV status; Kruskal–Wallis test used for all other comparisons among the three infection groups, within a given age category. Not significant (NS).

−0.168, P = 0.030, LRA) (Fig. 3). The percentage of monocytes making induced IL-8 did not differ significantly by the degree of malaria parasitemia; this was true for both children and adults (Fig. 3). Unlike monocytes, the percentage of lymphocytes spontaneously producing IL-8 did not differ between parasitemic chil-

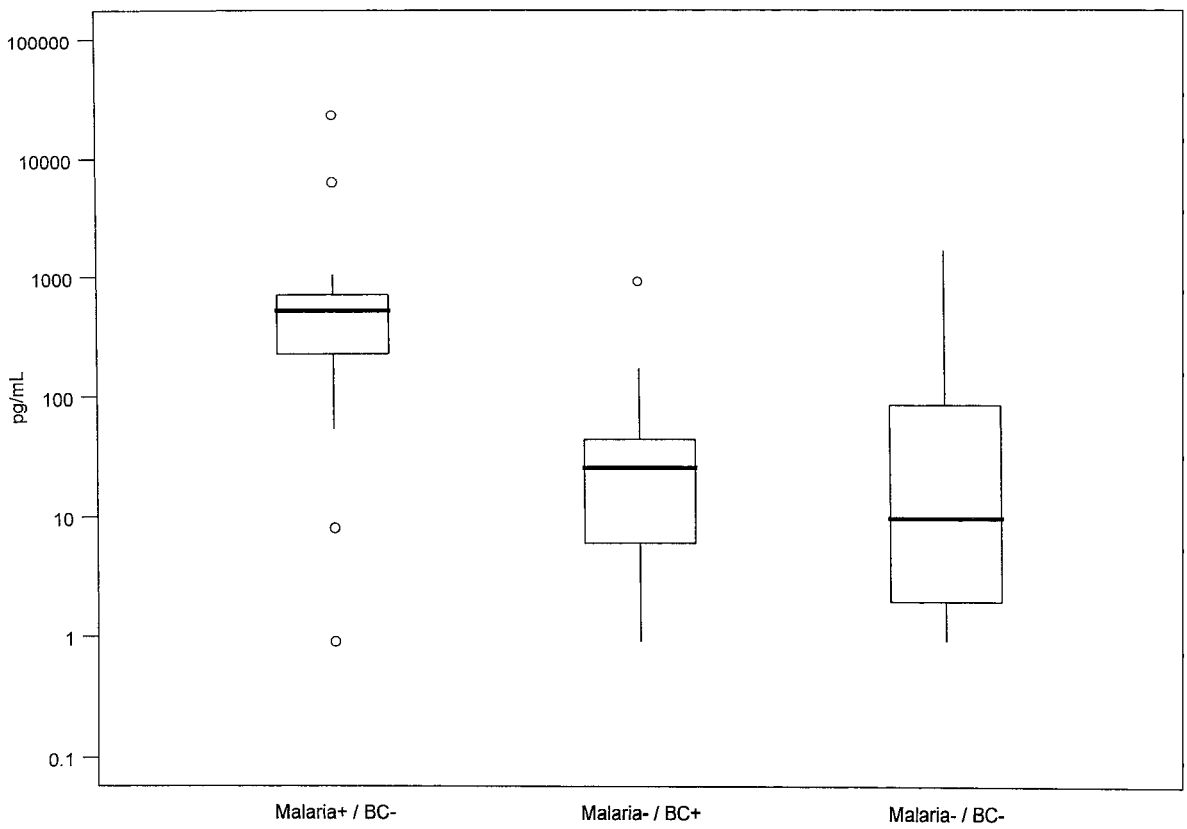

FIG. 1. Serum IL-10 levels, by patient group, including both pediatric and adult participants, with a logarithmic scale in picograms per milliliter. Boxes include medians (lines) and values between the 25th and 75th percentiles for the following participant groups: malaria$^+$/BC$^-$ (n = 24), malaria$^-$/BC$^+$ (n = 18), and malaria$^-$/BC$^-$ (n = 37). Lines extend to the farthest value within ±1.5× the interquartile range from the 25th and 75th percentiles; outliers are presented as circles. BC, blood culture.

TABLE 2

Parameters Varying Significantly by Malaria/Blood Culture (BC) Status, by Age Group[a]

Parameter	Medians			*P* values[b], Malaria$^+$ vs.	
	Malaria$^+$/BC$^-$	Malaria$^-$/BC$^-$	Malaria$^-$/BC$^+$	BC$^-$	BC$^+$
Children (<13 years old)	(n = 12)	(n = 114)	(n = 20)		
% spontaneously making IL-6					
All CD3$^+$ lymphocytes	1.6	20.5	19.3	0.002	0.014
CD3$^+$CD8$^+$ lymphocytes	1.4	33.8	28.8	0.003	0.047
Adults (≥13 years old)	(n = 24)	(n = 96)	(n = 40)		
% of lymphocytes					
Spontaneously making IL-4	0.9	1.6	3.2	0.015	0.003
Making induced IL-6	0.3	0.5	0.7	0.025	0.002

[a] Various data missing for various individuals. Three patients with both positive smears and positive blood cultures were excluded from the above table and all analyses. Twenty healthy adult control participants, described in text, also had malaria smears done; 2 were smear positive. Data included only if statistically significant for that age group.

[b] Wilcoxon tests.

dren and adults, nor did it vary with level of parasitemia (Fig. 4). However, for both children and adults, the percentage of lymphocytes making induced IL-8 increased significantly in relation to increasing parasitemia (r_s = 0.53, P = 0.002; b = 0.172, P = 0.011, LRA). This reflects the lower range endpoint being

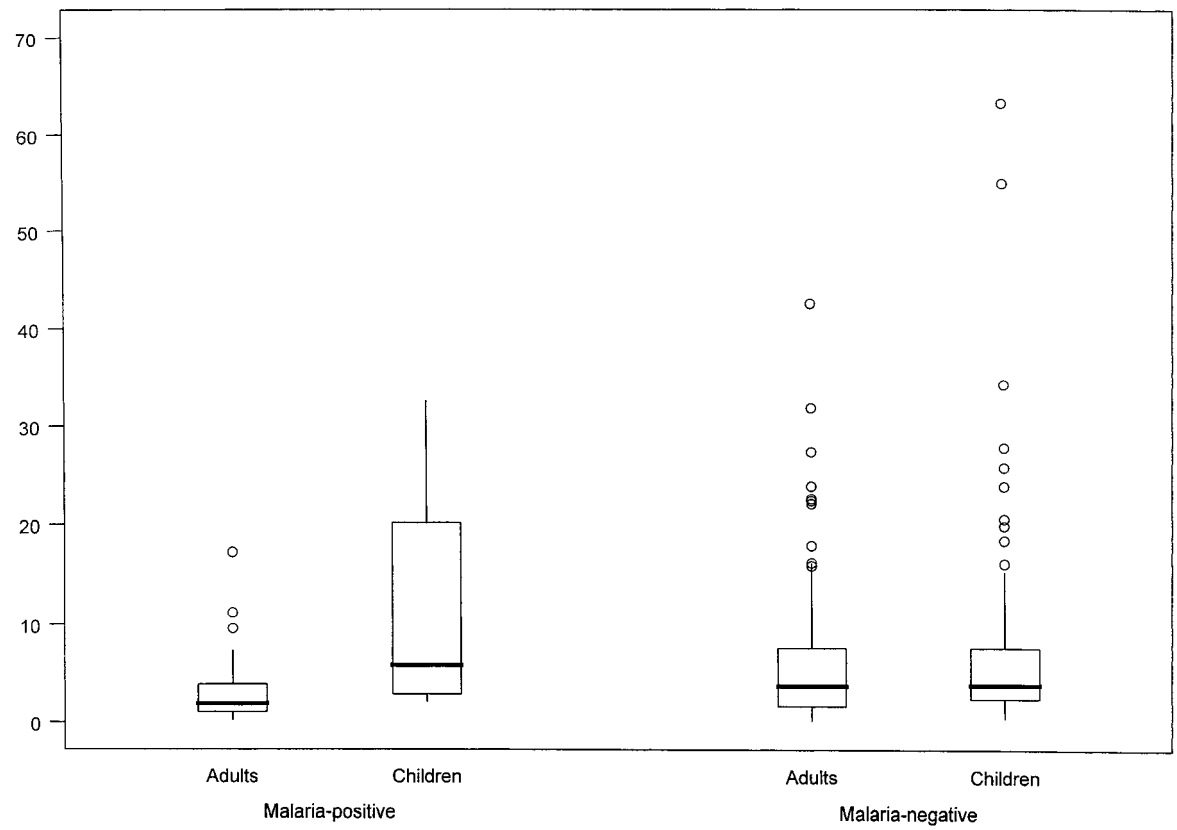

FIG. 2. Percentage of monocytes spontaneously producing IL-8, by age group and malaria status. Boxes include medians (lines) and values between the 25th and 75th percentiles (malaria-positive: adults, n = 26; children, n = 12, malaria-negative: adults, n = 151; children, n = 135). Lines extend to the farthest value within ±1.5× the interquartile range from the 25th and 75th percentiles; outliers are presented as circles.

TABLE 3
Immune Parameters Significantly Associated with Age Group[a]

Parameter	Stimulation status[b]	Median percentages: Adults with malaria (n's = 25–26)	Median percentages: Children with malaria (n's = 11–12)	P values[c]
Lymphocytes producing				
IL-6	S	0.6	2.2	0.008
IL-6	I	0.2	2.4	0.002
Both IL-6 and IFN-γ	S	0	0.1	0.003
Both IL-6 and IFN-γ	I	0	0.4	0.002
$CD3^+$ lymphocytes producing				
Both IL-6 and IFN-γ	I	0.1	3.0	0.003
$CD3^+CD8^-$ lymphocytes producing				
IL-6	I	0.2	2.9	<0.001
Both IL-6 and IFN-γ	I	0	1.3	<0.001
$CD3^+CD8^+$ lymphocytes producing				
IL-8	I	2.6	5.6	0.003
Both IL-6 and IFN-γ	S	0	0.2	0.002
Both IL-6 and IFN-γ	I	0.1	3.6	0.002

[a] Statistically significant differences between adults and children were present in those with and without malaria. Data provided are for those with malaria.
[b] Cytokine production was spontaneous (S) or induced (I).
[c] Wilcoxon tests, comparing adults with malaria to children with malaria. Only variables with $P < 0.01$ are included.

higher at levels 3+ and 4+ but is even more influenced by several adults and children with 3+ to 4+ parasitemia who had high percentages of lymphocytes producing IL-8 (Fig. 4).

HIV and Malaria Parasitemia

Only two parasitemic children were HIV^+; both had 1+ smears. For adults, no negative relationship was found between HIV serostatus or severity of HIV infection, as indicated by viral RNA levels and presence or level of malaria parasitemia (179,941 viral copies/mL, $n = 8$, for those with malaria; 588,544 copies/mL, $n = 16$, for those with positive blood cultures; and 248,276 copies/mL, $n = 23$, for those with negative malaria smears and blood cultures, not significant [NS]; data by level not shown). HIV-infected adults with parasitemia had less severe HIV immunosuppression, as represented by the percentage of lymphocytes expressing CD4 (14%, $n = 12$), than did other blood-culture-negative patients (7%, $n = 68$) ($P < 0.001$) and blood-

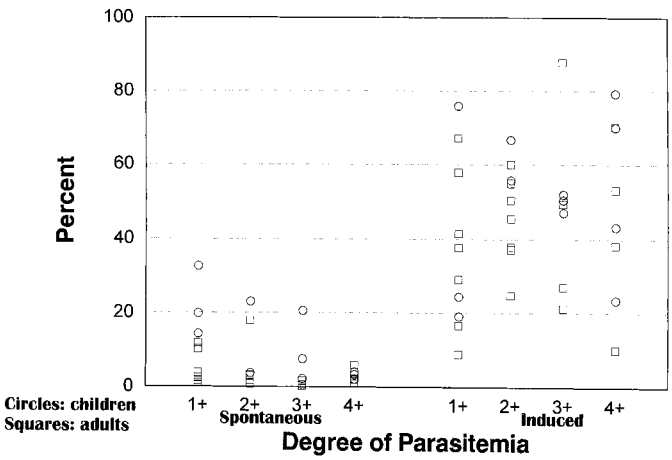

FIG. 3. Percentage of monocytes producing IL-8, by degree of malaria parasitemia and age group. Data are provided for spontaneous and induced production. Individual malaria patient values are charted (adults, $n = 22$; children, $n = 12$).

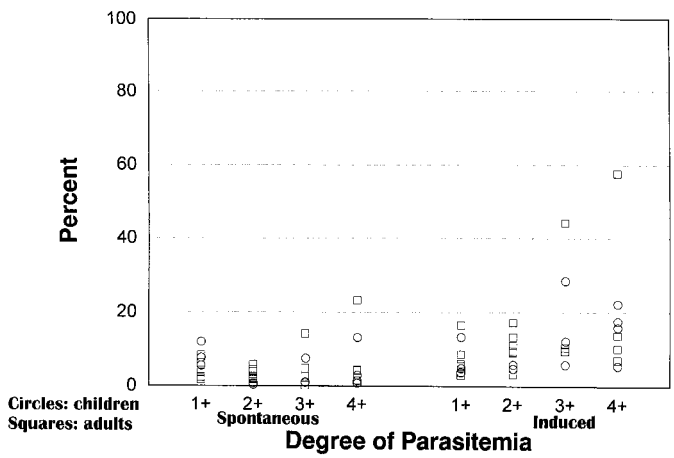

FIG. 4. Percentage of lymphocytes producing IL-8, by degree of malaria parasitemia and age group. Data are provided for spontaneous and induced production.

culture-positive patients (4%, $n = 35$) ($P < 0.001$), the latter group being the most immunodeficient of the three groups. HIV seroprevalence was similar between parasitemic adults and controls (67%), as was their percentage of lymphocytes expressing CD4 (controls, 35%). However, HIV RNA levels were significantly lower for controls (median ≤200 copies/mL) than for each of the three groups of adult patients ($P < 0.001$ for each). HIV serostatus, age, and severity of HIV infection as represented by the percentage of lymphocytes expressing CD4 and by HIV RNA levels were all unrelated to the degree of parasitemia (data not shown). The trends associated with malaria, age group, and degree of parasitemia were present when HIV-negative patients were analyzed separately (data not shown), as well as when HIV status was included as an independent variable in LRAs.

DISCUSSION

Cytokines may be key determinants of malaria severity and outcome (1–9, 11, 19–23) and are thus potential targets for therapeutic interventions if their effects can be better understood. Some studies suggest that the balance between pro- (TNF-α, IFN-γ, IL-6, IL-8) and antiinflammatory (IL-4, IL-10) cytokines determines the degree of malaria parasitemia, level of anemia, clinical severity, presentation, and/or outcome (3, 19, 20, 24)—but the cytokines described vary (6, 7, 11, 20–24). Other studies suggest that the absolute levels of cytokines, not the balance between them, determine malaria outcome. Specifically, extreme levels of antiinflammatory, as well as proinflammatory, cytokines may be associated with heightened disease severity and mortality (4).

To our knowledge, ours is the first study to assess both serum and cell-specific cytokines in patients with malaria parasitemia. We did not assess the relationships between cytokines and clinical presentation and outcome, given our small study size. Rather, we assessed the relationships between cytokines and the presence and degree of parasitemia, a relatively objective disease parameter. Further, in addition to comparing parasitemic patients to healthy individuals, we compared them to other acutely ill, hospitalized patients, who might be expected to also have alterations in their cytokine profiles. Thus, we could better differentiate parameters specifically related to malaria parasitemia from those related to fever, infection, or severe illnesses in general.

We found several parameters to be higher in adult malaria patients compared to malaria-negative, healthy controls, including the percentage of lymphocytes making induced IL-6 and spontaneously making IL-4. However, these parameters were significantly lower in malaria patients compared to the other patient groups, suggesting that malaria was less likely to evoke IL-6 and IL-4 responses than were other infections or illness in general. Of all the cytokine parameters assessed, only the serum levels of IL-10 were significantly higher in patients with malaria parasitemia compared to patients with other infections. Other serum cytokines were somewhat correlated with IL-10 levels (data not shown) but none were significantly related to parasitemia.

IL-10 induces B cell proliferation, plasma cell differentiation, and immunoglobulin production, all essential to the development and maturation of antimalarial antibodies. These effects are expected of a type 2 cytokine (25). However, IL-10 has a number of effects other than the stimulation of humoral immunity. In an *in vitro* malaria model, IL-10 decreased the production of IL-6 and TNF-α (a strongly inflammatory cytokine); anti-IL-10 antibody produced the opposite effect (20). In other studies, IL-10 inhibited IL-6, IFN-γ, and TNF-α secretion and function (25–28). In addition, it decreased major histocompatibility class II expression on macrophages, leading to decreased antigen presentation (29). It was associated with a reduction in the production of oxygen radicals and nitric oxide intermediates (25, 27, 28). Indeed, IL-10 decreases the inflammatory response in so many ways that it might best be thought of as a regulatory cytokine, rather than a type 2 cytokine. IL-10 counteracts IL-12's strong pressure toward a type 1 profile (30) by not only inhibiting type 1 cytokines but also inhibiting IL-12 itself (31–35), thus shifting the response toward a type 2 profile. The IL-10 response may be particularly critical in malaria. In a murine malaria model, mice with a disrupted IL-10 gene had elevated IFN-γ responses and mortality upon malaria challenge, suggesting that IL-10 was key in survival (2). Our data support the presence of a strong IL-10 response in human malaria. However, the source of this serum IL-10 is unclear since there were no significant cellular IL-10 findings in our study.

IL-6 has only rarely been cited as specifically important in malaria (4, 7, 26). It plays a critical role in red blood cell metabolism, activating heme progenitors, inducing megakaryocyte maturation, and inducing iron transport to the liver and bone marrow, as well as stimulating acute phase reactants (36). Since parasitemia is associated with red cell destruction and heme/iron release into the blood stream, it is not surprising that the percentage of blood cells making IL-6 would be lower in patients with malaria than in other patients. These malaria patients probably had a surfeit of iron, especially since they did not appear to have a high prevalence of anemia (median hematocrit 32%, range 17–43%, obtained for 8 of the children and 16 of the adults). Further, as noted above, these malaria patients had high levels of IL-10, which inhibits IL-6 production (20, 26). Our adult malaria patients had a

lower percentage of lymphocytes making IL-4 than did the other patient groups. IL-4 is a type 2 cytokine that stimulates naive $CD4^+$ cells to produce type 2 cytokines and induces the proliferation and differentiation of B cells (37). That IL-4 production is not increased in these malaria patients supports that IL-10's primary role in malaria is to dampen the inflammatory response, not to stimulate antibody production.

Serum IL-10 and cellular IL-10 parameters were not related to the degree of parasitemia. The only immune findings related to the level of parasitemia were cellular IL-8 parameters and these results were limited to monocytes in the peripheral blood of the children and lymphocytes in the blood of a handful of participants. IL-8 is a proinflammatory, chemotactic chemokine for neutrophils and T cells and a neutrophil stimulant that is involved in both malaria and the outcome/severity of infectious diseases in general (38–40). It activates neutrophils to release lysosomal enzymes and induces them to adhere to the vascular endothelium. In one study, serum IL-8 levels were correlated with the malarial parasite count (39). With *in vitro* lipopolysaccharide stimulation, IL-10 is a powerful inhibitor of IL-8 production (38). However, we did not find any relationship between serum IL-10 levels and parasitemia nor were serum or cellular IL-10 parameters correlated with any IL-8 parameters (data not shown). Thus, we cannot currently explain the mechanism of these IL-8 trends. IL-8 is produced by an especially broad array of cells, including monocytes, T cells, fibroblasts, endothelial keratinocytes, hepatocytes, chondrocytes, neutrophils, and epithelial cells (40). It may be that our IL-8 findings reflect cellular interactions and balancing with some of the cell types that we did not examine.

The relationship between HIV and malaria has been variously described as inhibitory, synergistic, or nonexistent (41–43). Our study was not designed to address this issue; our patient groups are not representative of the general Malawian population in that they represent severely ill individuals requiring hospitalization. Consistent with this bias, we found that many of the nonparasitemic, hospitalized adult participants were HIV positive, had very low percentages of lymphocytes expressing CD4, and were admitted with opportunistic infections, including bloodstream infections (14). Although the interaction between HIV and malaria was not our central concern in these analyses, our data do provide information concerning potential relationships between HIV disease severity and *levels* of malaria parasitemia: No relationship was found between level of parasitemia and HIV disease or HIV disease severity, as represented by either the percentage of lymphocytes expressing CD4 or the HIV RNA levels.

Perhaps our most interesting finding was that the proinflammatory response to malaria differed between children and adults: the percentages of monocytes spontaneously producing IL-8 were higher in children than in adults and the range in these percentages was much wider. The age-related disparity decreased with increasing levels of parasitemia, was not found following *in vitro* cytokine induction with PMA and ionomycin, and was not as striking in nonparasitemic patients. An age-related difference was not found in the percentages of lymphocytes producing IL-8. The potential importance of this dissimilarity is underscored by our further finding that the percentage of lymphocytes making induced IL-8 was the only parameter associated with the degree of parasitemia in both adults and children. Further, a number of other cellular proinflammatory parameters were significantly higher in children than adults, with or without malaria. These included the percentages of various lymphocyte populations making IL-6, both IL-6 and IFN-γ, or IL-8. These age-related differences may reflect very specific ways in which children's immune responses to infection in general and to malaria in particular are less effective than that of adults. They may be involved in the relatively greater severity of malaria in parasitemic children.

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