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Elevated serum levels of IL-1ra in children with *P. falciparum* malaria are associated with increased severity of disease

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Abstract

Animal models suggest that cytokines and chemokines play a role in cerebral malaria (CM) pathogenesis, but levels of a number of cytokines and chemokines thought to be important in the pathogenesis of other infectious diseases are not well characterized in children with CM. Serum levels of granulocyte-colony stimulating factor (G-CSF), interleukin-1 receptor antagonist (IL-1ra), interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1) were measured in 77 children with CM, 70 children with uncomplicated malaria (UM) and 63 healthy community children (CC) in Uganda. Children with CM had elevated serum levels of IL-1ra and IL-8 as compared to children with UM (median levels in pg/ml, 11,891 vs. 6,510, P=0.05, and 63 vs. 41, P=0.01, respectively). Children with CM who died (n=4) had higher serum levels than survivors of IL-1ra (median levels in pg/ml, 65,757 v. 10,355, *P*=0.02), G-CSF (709 vs. 117, *P*=0.02), and MCP-1 (1,275 vs. 216, *P*=0.03) but not IL-8 (76 v. 62, *P*=NS). Elevated IL-1ra levels are associated with increased disease severity in children with malaria, and very elevated levels of IL-1ra, G-CSF and MCP-1 are seen in children who die of CM.

Keywords

cerebral malaria; interleukin-1ra; granulocyte-colony stimulating factor; interleukin-8; monocyte chemoattractant protein-1

1. INTRODUCTION

In children with cerebral malaria (CM), we have previously investigated serum levels of a number of pro- and anti-inflammatory cytokines and chemokines thought to be important in malaria pathogenesis. The cytokines and chemokines tested included interferon-gamma (IFN–γ), interleukin (IL)–1β, IL-6, IL-10, tumor necrosis factor-alpha (TNF–α), macrophage inflammatory protein (MIP)–1α, MIP–1β, and RANTES. We documented elevated levels of all of these cytokines in children with CM, except RANTES, for which levels were lower in

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children with CM [1]. We also documented elevated levels of IL–1β, IL-10 and IFN–γ and lower levels of RANTES in children with CM as compared to children with uncomplicated malaria (UM). Finally, we documented that elevated levels of IL-6, MIP–1β, IL-10 and IFN–γ and lower levels of RANTES were seen in children with CM who died, as compared to those who survived.

These findings gave us a picture of the relative importance of these cytokines and chemokines in children with CM as compared to children with UM and healthy community children. In light of these findings, we hypothesized that other cytokines and chemokines that are important in serious central nervous system (CNS) infections, such bacterial meningitis and viral encephalitis, might also be important in systemic manifestations of cerebral malaria. For this reason we investigated, in children with CM, serum levels of additional chemokines (monocyte chemoattractant protein-1 (MCP-1) [2], and interleukin-8 (IL-8) [3]), and pro-inflammatory (granulocyte-colony stimulating factor (G-CSF) [4]) and anti-inflammatory (interleukin-1 receptor antagonist (IL-1ra) [5,6]) cytokines that have been implicated in the pathogenesis of other serious CNS and systemic infections and that have been associated with malaria pathogenesis pathways in previous murine or human malaria studies [7–10].

2. MATERIALS AND METHODS

2.1. Study population and recruitment

The study was conducted at Mulago Hospital, Kampala, Uganda. Children 4–12 years of age were recruited as part of two studies assessing the complications of cerebral malaria. A total of 88 children with cerebral malaria (CM), 76 children with UM, and 100 community children (CC) without evidence of acute illness were recruited. Complete details of the study cohorts were previously reported [1]. Briefly, children with CM were enrolled if they were admitted to Mulago Hospital and met the WHO criteria for CM: coma, *Plasmodium falciparum* on blood smear, and no other cause for coma. Ugandan Ministry of Health national guidelines for drug treatment of cerebral malaria (quinine for 7 days) were followed. Children with uncomplicated malaria (UM) were enrolled from the Acute Care clinic or an outpatient malaria clinic at the hospital sponsored by the University of California, San Francisco (UCSF). Children were considered to have UM if they had signs and symptoms of malaria, *P. falciparum* infection on blood smear, and no evidence of malaria complications or other acute illness. Children with UM were treated according the Ugandan Ministry of Health guidelines in Acute Care clinic (chloroquine plus sulfadoxine/pyrimethamine) or with combination therapy at the UCSF outpatient clinic (either amodiaquine plus sulfadoxine/pyrimethamine or amodiaquine plus artesunate). Community children were recruited from the extended household areas of children with CM or UM. Community children and children with UM were recruited to be in the same age range (4–12 years) as children with CM, and a history and physical exam was performed to ascertain that the community children were healthy at the time of enrollment.

Blood samples of 2–5 ml were obtained by venipuncture with the initial blood draw for routine labs, prior to or as anti-malarial treatment was being given. The separated serum was pipetted into aliquots and frozen at −70°C until testing was performed. Serum samples from 77 children with CM and 70 children with UM were available for testing, and serum samples of 62 of the 100 community children were randomly chosen for testing. This allowed testing of all samples on a limited number of plates. Written informed consent was obtained from the parents or guardians of study participants before enrollment of study participants. Ethical approval for the study was granted by the Institutional Review Boards for Human Studies at Makerere University Faculty of Medicine, University Hospitals of Cleveland and Case Western Reserve University, Indiana Wesleyan University and the University of Minnesota.

2.2. Cytokine testing

Serum levels of cytokines (G-CSF, IL-1ra) and chemokines (IL-8, MCP-1) that are altered in other CNS infections (see Introduction) were measured at the University of Minnesota. Cytokine and chemokine levels were determined by suspension array technology (SAT) using the Luminex system (Austin, TX) and human cytokine-specific bead sets (R&D Systems, Minneapolis, MN). Results were interpolated from 5-parameter-fit standard curves generated with the relevant recombinant human proteins (R&D Systems). Samples were tested at a 1:8 dilution.

2.3. Statistical analysis

All statistical analysis was performed using Stata 9.1 (Stata Corporation, College Station, Texas). Cytokine levels across two groups were compared with the Wilcoxon rank-sum two-sample test, adjusted for multiple comparisons by the method of Bonferroni. Correlations between cytokine levels were assessed by Spearman's rank correlation. Assessment of the relationship between cytokine levels and mortality, controlling for other cytokine levels, was performed by logistic regression analysis.

3. RESULTS

3.1. Serum cytokine and chemokine levels in children with CM, UM and community children

Children with CM, as compared to children with UM, had higher levels of IL-8 and IL-1ra, but not MCP-1 or G-CSF. Children with CM, as compared to CC, had higher levels of G-CSF, IL-1ra, IL-8 and MCP-1 (Table 1). IL-1β levels, reported previously[1], are included for comparison with IL-1ra levels. The ratio of IL-1ra:IL-1β levels (assigning a value of 1 pg/ml to all IL-1β responses below the level of detection) in children with CM ranged from 10 to 1,200. The IL-1ra:IL-1β ratio did not differ significantly between children with CM and UM, but was higher in these children than in CC (Table 1).

Levels of all cytokines and chemokines measured correlated strongly with each other (Spearman's rho range 0.51 – 0.72, all $P<0.0001$). Levels of these cytokines also correlated positively and strongly with the 7 of 8 cytokines previously assessed in this cohort (IL-1β, IL-6, IL-10, IFN-γ, MIP-1α, MIP-1β, and TNF-α; Spearman's rho range 0.32 – 0.79, all $P<0.0001$), and negatively with RANTES (Spearmans' rho range −0.28 – −0.36, all $P<0.0001$), except for IL-8, which did not correlate significantly with RANTES (Spearman's rho, −0.10, $P=0.16$). Serum G-CSF levels but not IL-8 levels correlated with absolute granulocyte count (Spearman's rho 0.43, P<0.0001, and 0.11, P=0.13, respectively), while serum MCP-1 levels correlated negatively with absolute monocyte count (Spearman's rho −0.31, P<0.0001).

3.2. Serum cytokine and chemokine levels and mortality

Mortality from CM was low in this study, with death occurring in only 4 of the 77 children with CM (5.2%). Children with CM who died had higher serum levels than survivors of IL-1ra, G-CSF, MCP-1 and but not IL-8 (Table 2). In logistic regression analysis, with each cytokine level associated with increased mortality (IL-1ra, G-CSF, MCP-1) adjusted for the levels of other cytokines, only levels of IL-1ra correlated with an increased risk of mortality (OR 1.00003, 95% CI 1.0000, 1.00005, $P=0.05$).

4. DISCUSSION

In the present study, IL-1ra and IL-8 levels were elevated in children with CM as compared to UM, and levels of IL-1ra, G-CSF and MCP-1, but not IL-8, were higher in children with CM who died. IL-1ra was the only cytokine or chemokine tested that was elevated in children with CM compared to children with UM and also elevated in children with CM who died as

compared to survivors. The progressive increase in IL-1ra levels from CC to UM to CM children and the very elevated IL-1ra levels in children who died suggest that IL-1ra may play a role in disease severity in *P. falciparum* malaria.

IL-1ra, unlike the other cytokines/chemokines tested in this study, is a potent anti-inflammatory cytokine. It regulates function of IL–1β, a pro-inflammatory cytokine. In bacterial infections, one study suggested that IL-1ra effectively counteracts excessive inflammation, and therefore decreases morbidity and mortality [11]. However, a recent study documented association of elevated IL-1ra or IL-1ra/pro-inflammatory cytokine ratios in individuals with meningococcal infections with severe disease and septic shock [5], suggesting that an overly vigorous anti-inflammatory response may dampen necessary inflammatory control of infection in this disease. The high levels of IL-1ra in CM, the progressive increase in levels from CC to UM to CM, and the very elevated levels in children who die of CM, all suggest that IL-1ra may also be a factor in severity of disease in *P. falciparum* infection. A previous study in the Gambia by Jakobsen also documented elevated levels of IL-1ra in children with CM as compared to mild or asymptomatic malaria infection, further supporting the association of elevated IL-1ra levels with disease severity in malaria [9]. In that study, reported levels of IL-ra in children with CM were lower (median IL-1ra level, 2,350 pg/ml in CM survivors) than in the present study (median IL-1ra level, 10,355 pg/ml in CM survivors). Direct comparison of levels is difficult, however, because of the different methods (ELISA in the study by Jakobsen et al, SAT in the present study) and standards used in the two studies. In the Gambian study, there was no difference in serum IL-1ra levels in children with CM who died as compared to those who died, while in the present study, children with CM who died had higher levels of IL-1ra than survivors. Differences between the studies, including the higher mortality rate in the Gambian study as compared to the present study (15% vs. 5.2%) and the lower median age of children in the Gambian study as compared to the present study (36 months vs. 60 months), might in part explain the differences in study results. Alternatively, genetic differences in IL-1ra receptors or genes between the two populations might affect IL-1ra production differently in children with CM in West African vs. East African populations. Although studies in Thailand [12] and Ghana [13] have not demonstrated an association between IL-1ra polymorphisms and disease severity within the populations, differences might still exist between populations that affect IL-1ra responses and disease severity. In the present study, IL-1ra levels also remained associated with mortality after adjustment for G-CSF and MCP-1 levels, although the association was not particularly strong (P=0.05). This may reflect a relatively weak independent association or the small sample size. No patient had physical exam or laboratory evidence of meningococcal disease or other bacterial infection, so we consider bacterial infection an unlikely explanation for the elevated IL-1ra levels. In a previous study, we also documented higher levels of the anti-inflammatory cytokine IL-10 in children with CM as opposed to UM, and higher levels of IL-10 in children with CM who died [1]. In that study, levels of the pro-inflammatory cytokines IL-6, IFN–γ and MIP–1β were also elevated in children who died, and in the present study, levels of the pro-inflammatory cytokines MCP-1 and G-CSF were elevated in children who died. Thus, anti-inflammatory cytokines, such as IL-1ra and IL-10, may be as important in severity of disease manifestations as pro-inflammatory cytokines, and the balance of pro- and anti-inflammatory cytokine activity is clearly important in disease severity in malaria. The present study supports the idea that measures that reduce pro-inflammatory activity in severe malaria may not necessarily result in improved outcomes if pro-inflammatory activity is reduced below the level important in control of the disease-causing organism. The activity and levels of specific cytokines may also be more important in disease pathogenesis and control in *P. falciparum* infection than the broader categorization of a “pro-inflammatory” or “anti-inflammatory” milieu.

G-CSF stimulates neutrophil production[10] and decreases neutrophil apoptosis[14], while IL-8 is a potent chemoattractant for neutrophils[15]. G-CSF but not IL-8 levels correlated with

peripheral absolute neutrophil count in the children in the present study. IL-8 levels, though elevated in CM, were anywhere from 100 to 1,000-fold lower than those seen in bacterial sepsis [16], and this may explain why IL-8 levels in CM did not correlate with peripheral neutrophil count. Alternatively, IL-8 in malaria may mobilize neutrophils to areas of sequestration rather than increase peripheral neutrophil counts. While some animal studies have suggested a role for neutrophils in control of rodent malaria [10], there is to date little data to support a role for neutrophils as important mediators of infection or disease in human malaria, so the contributions of G-CSF and IL-8 to protection from malaria infection and/or to malaria disease severity remain unclear.

Mouse models of CM with *P. bergheii* ANKA infection have suggested a role for MCP-1, an α-chemokine that attracts monocytes, in the pathogenesis of CM [8], but the role of MCP-1 in human subjects has been assessed previously only in placental malaria [7]. In the present study, we demonstrated that levels of MCP-1 are elevated in both UM and CM. Monocytes are a critical component of immunity to *P. falciparum*, particularly in antibody-dependent cellular inhibition (ADCI) [17], and our study findings are consistent with the idea that MCP-1 may play a role in host defense in UM and CM. However, like G-CSF and IL-1ra, high levels of MCP-1 were associated with death, highlighting again the potential importance of level of cytokine response to clinical outcome. The negative correlation between MCP-1 and absolute monocyte count suggests that MCP-1 is produced in response to a decreased monocyte count in the malaria-infected human host.

Although mechanisms by which elevated levels of IL-8, G-CSF and MCP-1 may assist in the control of *P. falciparum* infection and by which IL-1ra may modulate disease have been outlined above, it is possible that they are non-specific responses to infection and are not involved in control of infection. Similarly, it is always unclear in the case of children with CM who die whether elevated levels of multiple cytokines, producing a “cytokine storm”, are playing a role in mortality or simply markers of a final attempt by the immune system to counteract severe parasitic infection. In the present study, only IL-1ra levels demonstrated an increase across categories of disease severity in children with malaria and a further large increase in children with CM who died. The progressive increase in IL-1ra level according to disease severity up to and including death suggests that IL-1ra may be a contributor to *P. falciparum* malaria disease severity. However, the present study cannot assess causation, and the association between IL-1ra and increased disease severity and death may still be due to factors other than IL-1ra.

The low mortality rate in children with CM in this study, an outcome of considerable importance to us, nonetheless limited our ability to detect stronger associations between cytokine levels and death in children with CM. Future studies will be required to confirm our findings. The findings of this study are consistent with the idea that the balance between pro- and anti-inflammatory cytokine activity in response to *P. falciparum* that allows for control of infection without leading to worsening symptoms is a delicate one. The difficulty of adjusting this balance continues to confound efforts at adjunctive therapy for severe malaria. A broader understanding of the interplay between different cytokines and chemokines produced in response to *P. falciparum* infection and how they relate to disease severity may assist in development of more appropriately targeted interventions in the future.

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TABLE 1

Serum cytokine and chemokine levels in children with cerebral malaria (CM), uncomplicated malaria (UM) and community children (CC).

Cytokine	CM N=77 Median (range), pg/ml	UM N=70 Median (range), pg/ml	CC N=62 Median (range), pg/ml	P^a CM-UM	P^a CM-CC
G-CSF	126 (8 – 18,037)	92 (3 – 11,649)	29 (3 – 161)	NS	<0.0001
IL-1ra	11,891 (161 – 120,669)	6,910 (185 – 102,209)	629 (178 – 14,755)	0.05	<0.0001
IL-1β[b]	0 (0 – 1,063)	0 (0 – 5,795)	0 (0)	NS	<0.0001
IL-1ra: IL-1β[c]	4,310 (9 – 120,668)	3,343 (18 – 93,569)	629 (178 – 14,755)	NS	<0.0001
IL-8	63 (1 – 9,674)	41 (2 – 1,452)	29 (2 – 358)	0.01	0.008
MCP-1	216 (0 –14,253)	181 (31 – 13,866)	62 (15 – 260)	NS	<0.0001

[a] Wilcoxon rank-sum test.

[b] Previously published.

[c] IL-1ra: IL-1β ratio substitutes a value of 1 pg/ml for all values of IL-1β=0.

NS = not significant (P>0.05).

TABLE 2

Serum cytokine and chemokine levels in children with cerebral malaria (CM) who survived compared to those who died.

Cytokine	Survivors N=73 Median (range), pg/ml	Died N=4 Median (range), pg/ml	*P*[a]
G-CSF	117 (8 – 3071)	708 (312 – 18,037)	0.02
IL-1ra	10,355 (161 – 120,669)	65,757 (30,941 – 120,669)	0.02
IL-1ra: IL-1β	4,059 (9 – 120,669)	36,527 (639 – 89,399)	NS
IL-8	62 (1 – 844)	76 (56 – 9,674)	NS
MCP-1	216 (0 –3,597)	1,275 (214 – 14,253)	0.03

[a]Wilcoxon rank-sum test.

[b]IL-1ra: IL-1β ratio substitutes a value of 1 pg/ml for all values of IL-1β=0.

NS = not significant (P>0.05).