Tropical Medicine and International Health

VOLUME 2 NO 7 PP 673–679 JULY 1997

Cytokine levels during mild and cerebral falciparum malaria in children living in a mesoendemic area

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Summary Cell-mediated immunity and cytokines are probably involved in the pathogenesis of malaria. To investigate the role and the activity of different immune cells, we measured levels of tumour necrosis factor-(TNF-α), gamma interferon (IFN-γ) and several interleukins (IL-2, IL-4, IL-6 and IL-10) in children with mild (MM) and cerebral (CM) *Plasmodium falciparum* malaria and compared them with those of healthy children from Guadalupe – Lobata District, St. Tomé Island, where malaria is mesoendemic. Both groups of patients had significantly higher levels of IL-6, IL-10 and TNF-α than controls. For IL-2, IL-4 and IFN-γ we found no difference between the groups. However, 24 h after admission the levels of IL-10 and IL-6 were significantly higher in CM than in MM patients, although 7 days after treatment they returned to normal levels, similar to those found in control children. Therefore, TNF-α IL-6 and IL-10 increase during *Plasmodium falciparum* attacks in all children, not only in those with cerebral malaria. This finding suggests the activation of the monocyte/macrophage system during the early stage of clinical malaria.

keywords cytokines, *Plasmodium falciparum*, mild malaria, cerebral malaria, St. Tomé Island

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Introduction

The pathogenesis of *Plasmodium falciparum* malaria depends on many factors, including immunological ones. Although antibody-mediated responses are important (McGregor *et al*. 1956), cell-mediated immunity is involved not only in protection but also in the pathogenesis of disease, and some reports suggest that host immunological responses, especially those mediated through cytokine cascade, may determine disease severity (Clark *et al*. 1989). *Plasmodium* parasites are able to induce the production of several cytokines (Allan *et al*. 1995; Jakobsen *et al*. 1994) regulating the cellular immune response and possibly influencing the mechanisms of clinical disease (Grau *et al*. 1989; Kwiatkowski *et al*. 1990).

Activated monocyte/macrophage cells secrete predominantly tumour necrosis factor alpha (TNF-α), interleukin-6 (IL-6) and IL–10. TNF-α has been associated with the pathophysiology of malaria and correlated with disease severity (Clark & Chaudhri 1988; Clark *et al*. 1989; Grau *et al*. 1989; Kern *et al*. 1989; Kwiatkowski *et al*. 1990, 1993). However, conflicting results concerning the association between severity of malaria and TNF-α levels have been reported. T helper 1 (Th1) and T helper 2 (Th2) subsets are involved in the regulation of immune responses. A Th-type 1 response produces mainly IL-2, interferon (IFN-γ) and a cell-mediated inflammatory reaction, while a Th-type 2 response produces mainly IL-4 and stimulates antibody production (Mosmann & Coffman 1989). We measured plasma levels of TNF-(IFN-γ) IL-2, IL-4, IL-6 and IL-10 in children with cerebral malaria (CM) and mild malaria (MM) in St. Tomé Island

District to find out whether severe *P. falciparum* malaria is associated with a particular immune cell activity.

Patients and Methods

Study population

St. Tomé Island is a malaria-endemic area situated in the Gulf of Guinea. *P. falciparum* is the predominant parasite but mixed infections with *P. malariae*, *P. vivax*, and *P. ovale* have been described. In the 1980s, effective malaria control measures caused a sharp decrease in malaria transmission, and consequently a decrease in malaria morbidity and malaria immunity. However, these measures were interrupted in 1983/4, which caused a large malaria outbreak in 1985/86. Since then, *P. falciparum* transmission has stabilised (Baptista, 1996).

This study was carried out between November 1992 and November 1993 among children attending the village health centre of Guadalupe (Lobata District). Informed consent was obtained from the parents or guardians of the children recruited according to the criteria of mild (MM) and cerebral (CM) malaria of the World Health Organization (Warrel *et al*. 1990). After evaluation, all patients were admitted for parenteral therapy until day 7, when they were discharged.

All selected patients had only *P. falciparum* infection and had not taken antimalarial drugs before admission (negative Lelijveld and Kortmann urine test for 4-amino-quinolines) (Bruce-Chwatt *et al*. 1986). Patients positive for sickle-cell test (Gentilini 1992), for human immunodeficiency virus type I and type II (HIV I – ELISA, Wellcome; HIV II – ELISA, Quilaban) and for hepatitis B surface antigen (Hbs Ag – ELISA – Abbot), were excluded.

All children had a lumbar puncture at the time of admission. The cerebrospinal fluid was examined immediately, and cell count performed with standard counting chamber. Patients with more than 10 leukocytes/mm3 in the cerebrospinal fluid, or those with evidence of other severe infections, were excluded from the study. Children were considered to have CM if they were comatose (coma score of 2 or less on the Glasgow coma scale) (Rogers 1992), had detectable *P. falciparum* asexual forms in peripheral blood smears and had no other evident cause of illness. Thirty healthy children of the same age group were selected as controls: they were from the same community, had no detectable malaria parasites and were negative for HIV-I, HIV-II and Hbs Ag.

Sample collection

Blood samples were collected from each patient before treatment (D0), 24 h later (D1), and on the day of discharge (D7). The follow-up visit after an additional week (D14) included a brief clinical examination, a thick blood film for parasitaemia and a venous blood sample. At D0 plasma levels of IL-2, IL-4, IL-6, IL-10, IFN-γ and TNF-α were tested while at D1, D7 and D14 only levels of IL-2, IL-4, IL-6 and IL-10 were measured. Venous blood samples were collected by venopuncture into EDTA sterile containers (Monovette®KE, Sarstedt, Belgium). Less than 10 min after venopuncture, the blood was spun for 15 min at 500 rpm and at room temperature. Plasma was collected in 1 ml plastic containers and stored in liquid nitrogen until cytokine assays could be done at the Institute of Tropical Medicine in Antwerp (Belgium). Specimens were stored for no longer than 3 months.

Laboratory methods

Thick and thin blood smears, later stained with Giemsa, were collected regularly from the patients for parasite density until clearance. This was expressed as the number of asexual parasites/μl blood and was computed from the number of asexual parasites over the number of leukocytes in 100 high-power fields, assuming a leukocyte count of 8×10^9/l of blood.

The IFN-(IL-2, IL-4, IL-6, IL-10 and TNF-α concentrations in plasma were determined using a solid phase enzyme amplified sensitivity immunoassay performed on microtitre plates (*Kit Medgenix EASIA* – Medgenix – Fleurus, Belgium). The sensitivity (minimum detectable concentration) limits of these ELISAs were 0.03 IU/ml for IFN-γ, 0.1 IU/ml for IL-2, 2.0 pg/ml for IL-4, 2.0 pg/ml for IL-6, 1 pg/ml for IL-10 and 3.0 pg/ml for TNF-α. The assay is a double sandwich ELISA using monospecific polyclonal rabbit antibodies to purified recombinant cytokines. The plasma samples were thawed at room temperature and assayed in duplicate for each sample, according to the manufacturer's instructions. A standard curve was obtained colorimetrically (*Bio-Tek Microplate Reader,*

Table 1 Median of parasitaemia (range) and cytokines in children with cerebral ($n = 12$) or mild malaria ($n = 44$)(*), before treatment (D0), 24 hours after treatment (D1) and in healthy control children ($n = 30$). IFN-γ, IL-4 and TNF-α at D1 have been measured only in the cases of cerebral malaria

	Cerebral malaria			Mild malaria*	Control
Parasitaemia/cytokines	D0	D1	D0	D1	
Parasitaemia (parasites/μl)	62100 (35040–287520)	6680 (20–26560)	34720 (5000–90080)	1140 (0–49920)	—
IFN-γ (IU/ml)	1.2 (1.2–1.3)	—	1.2 (1.2–1.3)	—	1.2 (1.2–1.3)
IL-2 (IU/ml)	1.5 (1.5–1.5)	1.5 (1.5–1.5)	1.5 1.5–1.5)	1.5 1.5–1.5)	1.5 1.5–1.5)
IL-4 (pg/ml)	22.7 (22.3–23.2)	23.4 (22.5–23.9)	22.8 (22.6–23.3)	—	23.1 (22.9–23.5)
IL-6 (pg/ml)	65.4 (52.9–78.4)	107.7 (65.8–171.1)	53.2 (46.6–65.3)	52.7 (48.3–60.3)	47.0 (45.8–48.6)
IL-10 (pg/ml)	229.8 (62.3–614.1)	226.4 (74.1–449.2)	102.1 (44.0–216.8)	43.6 (28.3–71.1)	21.9 (18.7–24.0)
TNF-α (pg/ml)	43.9 (39.8–61.1)	—	49.8 (42.7–57.7)	—	33.3 (33.3–33.9)

*In the case of IFN-γ, IL-4 and TNF-α we did not measure the plasma samples at D1 for mild malaria (in this case n = 9). IL, interleukin; TNF-α, tumour necrosis factor; IFN-γ (interferon gamma)

Ety-System, Fast Reader) and the level of cytokines in the plasma determined from the curve.

Treatment

All children with malaria were admitted for parenteral therapy. Each patient was weighed and a loading dose of quinine dihydrochloride (20 mg salt/kg in a 4 h perfusion containing 5% dextrose) was given, followed by perfusions of 10 mg salt/kg every 8 h until tablets could be given. This treatment lasted 7 days.

Statistical analysis

Kruskal–Wallis and Spearman rank tests were used for analysis, with a $P < 0.05$ level of significance. Cytokine levels were represented using box-and-wiskers plots.

Results

Population

Fifty-six children, 44 with MM [16 males and 28 females (median age: 9, range 4–14) and 12 with CM [7 males and 5 females (median age: 5, range 4–9), were recruited. The median duration of fever before attending the health centre was 24 h (range 12–72 h). The clinical outcome after treatment was good in all cases of MM. In the CM group, 3 patients (25%) died within 24 h of admission. None of the surviving patients had sequelae at the time of discharge.

The median age of the control group ($n = 30$: 14 males and 16 females) was 9.5 years (range 4–14). There was no association between cytokine levels and age of the children.

Parasitaemia

On admission, levels of parasitaemia ranged from 5000 to 287520 parasites/μl, and the median parasite density was significantly higher in the CM group (Table 1) than in the MM group ($P < 0.001$). The longest time for parasite clearance was 96 h; no recrudescence was observed within the following 14 days of observation. At admission, parasite density was not higher in patients who died of CM compared to those who survived ($P > 0.05$).

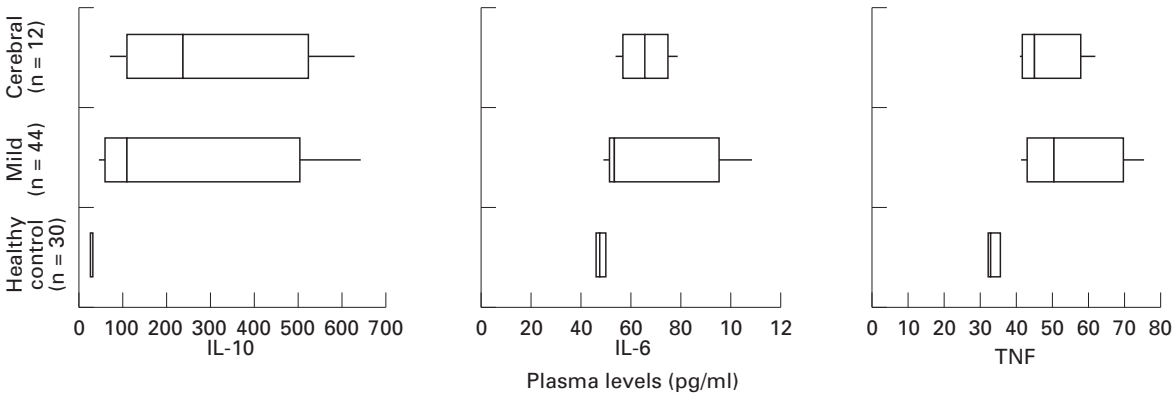

Figure 1 Plasma levels of interleukin-10 (IL-10), IL-6 and TNF-α in children with cerebral ($n = 12$) or mild ($n = 44$) malaria and healthy children (Controls: $n = 30$). Boxes represent 25th and 75th percentiles, with median (solid vertical lines). Horizontal lines indicate 5th and 95th percentiles.

Association cytokines – malaria

At D0 sick children had significantly higher levels of IL-6, IL-10 and TNF-α than healthy children ($P < 0.001$) (Table 1 – Figure 1), but no difference between CM and MM cases and between CM children who died and those who survived was found. MM children had higher levels of TNF-α than CM children (Figure 1), while these had slightly higher levels of IL-10 or IL-6 than MM children. However, these differences were not statistically significant. Plasma levels of IL-2, IFN-γ and IL-4 were similar in all groups of children (Table 1). Parasite density was not associated with cytokine levels.

At D1 malaria patients had higher levels of IL-6 and IL-10 than control children and CM children had significantly higher levels of IL-6 ($r = 0.51$, $P < 0.001$) and IL-10 ($r = 0.21$, $P < 0.001$) than MM children (Table 1). Levels of IL-2 and IL-4 ($n = 12$) were similar at D1 for CM and MM cases. At D7 (Figure 2) and D14

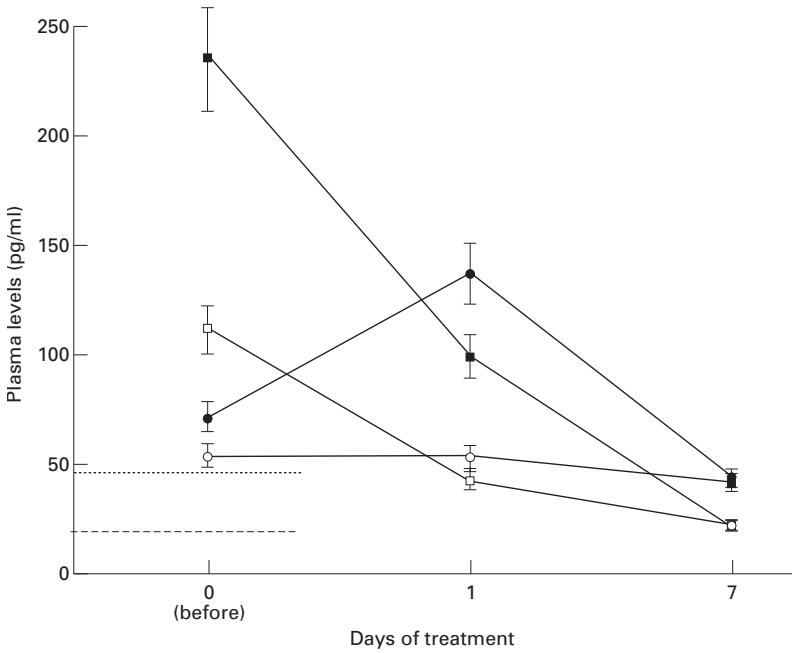

Figure 2 Plasma levels of IL-10 (□, ■) and IL-6 (○, ●)in children with cerebral (closed symbols, $n = 9$) or mild malaria (open symbols, $n = 21$), before treatment, after 24 h and after 7 days. The plasma levels 14 days after (not represented) was the same at D7.

levels of IL-2, IL-4, IL-6 and IL-10 were similar to those found in healthy controls.

Discussion

We tried, by studying children with mild or cerebral malaria, to find out whether cytokine patterns which reflect the activity of different cells of the immune system, including monocytes/macrophages and Th-type 1 and Th-type 2 lymphocyte subsets, are associated with the severity of *P. falciparum* malaria.

Levels of IL-10 were significantly higher in children with clinical malaria than in healthy controls. Previous reports have found higher IL-10 plasma levels in adults with *P. falciparum* malaria (Peyron *et al.* 1994; Deloron *et al.* 1994). However, to our knowledge, this is the first report of increased levels of IL-10 in children from a malaria-endemic area. As the BCRF1 (viral IL-10) was not measured, the possibility that IL-10 was of viral origin (Burdin *et al.* 1993) cannot be ruled out, although the assay used had a low level of cross-reaction ($< 0.2\%$) with BCRF1. The monocyte/macrophage system produces IL-10, but at a later stage than other cytokines (De Waal Malefyt *et al.* 1989; Stips & De Waal Malefyt 1992). Therefore, because children with malaria attended the health centre several hours after the onset of disease, it is likely that the increased levels of IL-10 in our patients were caused by the monocyte/macrophage activity. Although we found no statistically significant association with severity of disease, our data suggest that children with CM might have higher plasma levels of IL-10 than those with a mild attack. At D1 circulating IL-10 decreased in MM patients, but the high levels persisted in CM children and the difference between these two groups of patients was statistically significant ($P < 0.001$). This confirms what was reported by Peyron *et al.* (1994) in adults and suggests a link between plasma levels of IL-10 and clinical symptoms but not with parasite density.

TNF-α and IL-6 were higher in malaria patients than in healthy controls. TNF-α was also higher in MM than in CM patients, although this last association was not statistically significant. This is in contrast with the findings of several authors (Clark & Chaudhri 1988; Clark *et al.* 1989; Grau *et al.* 1989; Butcher *et al.* 1990; Nicolas *et al.* 1994) who suggested that these cytokines are involved in the pathogenesis of CM malaria and predictive of fatal outcome. TNF-α is released intermittently, at the time of schizont rupture, which in patients with natural infection usually is not synchronized (Kwiatkowski *et al.* 1989). Thus it is possible that the higher levels of TNF-α found in MM patients is just a chance finding, reflecting the time of schizont rupture and not disease severity. However, MM children had higher TNF-α and IL-6 levels and recovered rapidly with treatment, indicating that, at least in our study, these are not markers of severity. This should be taken with caution as the number of cases involved is small (12 patients in the group of CM with 3 fatal cases).

Levels of IL-2, IL-4 and IFN-γ were similar in the 3 groups. This contrasts with previous results reporting an association between lymphocyte-derived cytokines and malaria infection (Chizzolini *et al.* 1990; Riley *et al.* 1991; Jakobsen *et al.* 1994). The time between onset of disease and recruitment should be considered to explain this discrepancy: IL-2 has a serum half-life of 6–9 min and rapidly disappears from circulating blood (Lotze *et al.* 1985). Furthermore, cytokine production may precede the onset of clinical manifestations (Harpaz *et al.* 1992), may vary with parasite stage (Allan *et al.* 1995) or with parasite densities (Kwiatkowski *et al.* 1990) and may be localized. In malaria-infected mice reactive lymphocytes disappear from the blood, but their number increases significantly in the spleen (Weiss 1990). Therefore, circulating levels of IFN-γ and IL-4 may not reflect local cytokines activity during malaria infection.

The role of the cytokine network in malaria is still unknown and interpreting the different level of cytokines in the plasma is difficult. In humans, studies investigating the relationship between cytokines and acute *P. falciparum* malaria infection may be limited by several problems as the difficulty of obtaining an appropriate control group, the lack of international standards for cytokine assays (Kossodo *et al.* 1995) and the short half-life of some.

P. falciparum parasitaemia may be cleared by phagocytosis. High concentrations of proinflammatory cytokines, such a TNF-α and IL-6, seem to be deleterious for the host, although at low doses these may promote the clearance of parasites (Peyron *et al.* 1994). The presence of circulating IL-6 and TNF-α together with IL-10 in the early stage of malaria may suggest an activation of monocytes/macrophages. The beneficial (by reducing the inflammatory response) or detrimental

(by decreasing the cellular response) role of IL-10 during malaria attacks remains to be clarified. This could have important consequences, as the modulation of these mediators during acute disease could lead to a novel approach to the treatment of malaria.

Acknowledgements

We thank public health nurse Mário Gomes for his collaboration. I would also like to thank Drs Umberto D'Alessandro and Jean Claude Dujardin for their helpful comments during the preparation of this paper.

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