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ORIGINAL ARTICLE

Sequential Serum Cytokine Levels of TNF-Alpha, IL-4 and IL-12 are Associated with Prognosis in *Plasmodium falciparum* Malaria

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Abstract We investigated the prognostic role of TNF-alpha, IL-4 and IL-12 in a clinically well defined group of *Plasmodium falciparum* infected patients ($n = 32$) sequentially from Day 0 to Day 10 with a 2 day interval along with a control group of 16 healthy volunteers of same range of age and sex. Infection with malaria is often fatal because mitochondria are unable to generate enough ATP to maintain normal cellular function. ATP deficiency arises in malaria due to an inability of mitochondria through the effects of inflammatory cytokines on their function, to utilize available oxygen. In our study TNF-alpha and IL-12 levels were significantly elevated but IL-4 level showed persistent decline in Day 0, but subsequent measurement in Day 2, 4, 6, 8 and 10 showed persistent decline in levels of TNF-alpha and IL-12, an elevation in IL-4 levels which were associated with disease prognosis of the infected patients. These results again provide evidence that cytokines are very much a dominant partner in malaria pathogenesis with a specific prognostic role.

Keywords Cytokines · TNF-alpha · IL-12 · IL-4 · Pf malaria · Day 0–10

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Introduction

Malaria is possibly the most serious infectious disease of humans, infecting 5–10 % of the world's population, with 300–600 million clinical cases and more than 2 million deaths annually [1]. Cerebral malaria (CM) is a deadly complication of *Plasmodium falciparum* infection, One of the earliest events in CM pathogenesis appears to be a mild increase in the permeability to protein of the blood–brain barrier. Recent studies have shown a role for $CD8^+$ T cells in mediating damage to the microvascular endothelium and this damage can result in the leakage of cytokines, malaria antigens and other potentially harmful molecules across the blood–brain barrier into the cerebral parenchyma. This, in turn, leads to activation of microglia and apoptosis of astrocytes. The role of hypoxia in the pathogenesis of CM, with particular reference to the local reduction of oxygen consumption in the brain as a consequence of vascular obstruction, to cytokine-driven changes in glucose metabolism, and to cytopathic hypoxia. There are two major theories to explain the pathogenesis of human CM. The 'mechanical obstruction' hypothesis suggests that CM is a consequence of the adherence of parasitized red blood cells (PRBCs) to the cerebral microvascular endothelium, leading to vascular obstruction and cerebral hypoxia [2]. The 'cytokine' theory assigns a central role to an immunopathological process

involving cytokines such as interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α) [3].

The pathogenic manifestations during a malaria crisis are due to proinflammatory cytokines released by T cells and macrophages in response to malaria parasites and their products, including glycosylphosphatidylinositol (GPI) moieties [4], malaria pigment [5], and plasmodium-derived nitric oxide synthase (NOS)-inducing factor [6].

The first characterized parasite induced cytokine was TNF-alpha induced in macrophages by erythrocytes infected by plasmodium, malarial pigment and certain glycolipids such as GPI moiety. It has been shown that GPI moiety induces NOS in macrophages and activates endothelial cells by tyrosine-kinase mediated signal transduction. IL-12 is a potent immunomodulatory cytokine which not only increases cell-mediated immune response but also affects humoral immunity by inducing isotype switching through both interferon-γ dependent and independent mechanisms. IL-4 is produced by activated T cells of the Th-2 subtype and mast cells, it has been seen to be involved in the activation of CTL, NK cells and macrophages. Interleukin 4 and Th-2 subtype cells are important component of immune response stimulating growth of Th-2 and inhibiting Th-1 response by depressing the production of interferon-γ [7].

The main goal of the study was to associate the sequential cytokine levels of TNF-alpha, IL-12 and IL-4 with the prognosis of *P. falciparum* patients.

Materials and Methods

The study was conducted in S.C.B Medical College Hospital Cuttack the largest tertiary referral government hospital in Odisha. Inclusion and classification of each case were based on symptoms, physical signs and laboratory results of malaria at the time of first presentation.

P. falciparum malaria was established by microscopic diagnosis of *P. falciparum* parasites in the peripheral blood and clinical signs according to the WHO criteria: evidence of neurological compromission (prostration, lethargy), gastrointestinal symptoms, severe anaemia (Hb <6 g/dl), hyperparasitaemia corresponding to Ep $> 5 \times 10^5$ or 5 %, acidosis with respiratory distress, oliguria, cardiovascular shock, jaundice, diffuse hemorrhages. This study included 32 patients of *P. falciparum* malaria admitted in medicine ward of SCB Medical College.

As a control group for cytokine determination 16 healthy volunteers from Cuttack of same range of age and sex were also included in the study. All volunteers enrolled as control group were negative at the thick-smear examination for *P. falciparum* and *P. vivax*, without febrile episodes during last 6 months and without signs of anaemia. (Hb >10 g/dl). The above study was approved by Institutional Ethics Committee, S.C.B Medical College Cuttack, and subjects gave informed consent to the work.

Detection of Parasitaemia and Sample Collection

For detection of parasitaemia, a calibrated thick-smear technique was used, with standard Giemsa staining. The blood samples were collected for immunological assessment in sterile tubes. All the samples were centrifuged and serum was refrigerated at −40 °C and was sent to the Laboratory of Department of Biochemistry, S.C.B. Medical Cuttack for the determination of IL-12. IL-4 and TNF-alpha.

Cytokine Assays

Serum samples were analyzed for IL-12, IL-4 and TNF-alpha using enzyme-linked immunosorbent assay (ELISA) obtained commercially (Ray Biotech Inc, 3607 Parkway Lane, Suite 200, Norcross GA 30092)The assays were performed according to the manufacturer's protocol. Each plate included a standard curve and known positive and negative controls. Absorbance was read against a blank at 450 nm using a microtiter ELISA reader.

Statistical Analysis

Serum cytokine concentration were determined in duplicate and expressed as mean ± SE of the mean. Comparisons between groups were made using Z test with statistical significance set at SE value of 1 % level of significance (SE = standard error of mean).

Results

In Day 0 (Table 1) mean levels of TNF-alpha were 955 ± 261.33 pg/ml, compared to IL-12 was 293.12 ± 94.64 pg/ml and IL-4 were 2.35 ± 0.64 pg/ml.

In Day 2 (Table 2) mean levels of TNF-alpha were 307.50 ± 136.40 pg/ml, compared to IL-12 was found to be 160.63 ± 20.81 pg/ml and IL-4 were 3.2 ± 0.13 pg/ml.

In Day 4 (Table 3) mean levels of TNF-alpha were 155 ± 23.66 pg/ml, compared to IL-12 was found to be 117.5 ± 8.16 pg/ml and IL-4 were 3.7 ± 0.11 pg/ml.

In Day 6 (Table 4) mean levels of TNF-alpha were 103.63 ± 10.20 pg/ml compared to IL-12 was found to be 89.25 ± 5.66 pg/ml and IL-4 were 4.24 ± 0 0.233 pg/ml.

In Day 8 (Table 5) mean levels of TNF-alpha were 82.25 ± 4.89 pg/ml, compared to IL-12 was to be 69.75 ± 7.37 pg/ml and IL-4 were 4.8 ± 0.17 pg/ml.

Table 1 Serum cytokine levels of TNF-alpha, IL-4 and IL-12 in *P. falciparum* patients ($n = 32$) on Day 0

Parameters	Day 0 Mean	Day 0 SD	HC Mean	HC SD	Z Calculated value	Test SE value of 1 % level of significance
TNF-alpha (pg/ml)	955	261.33	42.9	13.5	954.07	2.58
IL-12 (pg/ml)	293.12	94.64	49.8	11.59	14.33	2.58
IL-4 (pg/ml)	2.35	0.64	6.06	1.32	10.6	2.58

Table 2 Serum cytokine levels of TNF-alpha, IL-4 and IL-12 in *P. falciparum* patients ($n = 32$) on Day 2

Parameters	Day 2 Mean	Day 2 SD	HC Mean	HC SD	Z Calculated value	Test SE value of 1 % level of significance
TNF-alpha (pg/ml)	307.5	136.40	42.9	13.5	10.87	2.58
IL-12 (pg/ml)	160.63	20.81	49.8	11.59	23.68	2.58
IL-4 (pg/ml)	3.2	0.13	6.06	1.32	8.64	2.58

Table 3 Serum cytokine levels of TNF-alpha, IL-4 and IL-12 in *P. falciparum* patients ($n = 32$) on Day 4

Parameters	Day 4 Mean	Day 4 SD	HC Mean	HC SD	Z Calculated value	Test SE value of 1 % level of significance
TNF-alpha (pg/ml)	155	23.66	42.9	13.5	20.88	2.58
IL-12 (pg/ml)	117.5	8.16	49.8	11.59	20.89	2.58
IL-4 (pg/ml)	3.7	0.11	6.06	1.32	7.2	2.58

Table 4 Serum cytokine levels of TNF-alpha, IL-4 and IL-12 in *P. falciparum* patients ($n = 32$) on Day 6

Parameters	Day 6 Mean	Day 6 SD	HC Mean	HC SD	Z Calculated value	Test SE value of 1 % level of significance
TNF-alpha (pg/ml)	103.63	10.20	42.9	13.5	15.86	2.58
IL-12 (pg/ml)	89.25	5.66	49.8	11.59	12.89	2.58
IL-4 (pg/ml)	4.24	0.233	6.06	1.32	5.51	2.58

Table 5 Serum cytokine levels of TNF-alpha, IL-4 and IL-12 in *P. falciparum* patients ($n = 32$) on Day 8

Parameters	Day 8 Mean	Day 8 SD	HC Mean	HC SD	Z Calculated value	Test SE value of 1 % level of significance
TNF-alpha (pg/ml)	82.25	4.89	42.9	13.5	11.31	2.58
IL-12 (pg/ml)	69.75	7.37	49.8	11.59	6.29	2.58
IL-4 (pg/ml)	4.8	0.17	6.06	1.32	3.89	2.58

Table 6 Serum cytokine levels of TNF-alpha, IL-4 and IL-12 in *P. falciparum* patients ($n = 32$) on Day 10

Parameters	Day 10 Mean	Day 10 SD	HC Mean	HC SD	Z Calculated value	Test SE value of 1 % level of significance
TNF-alpha (pg/ml)	60.87	7.82	42.9	13.5	4.92	2.58
IL-12 (pg/ml)	47.5	8.27	49.8	11.59	0.71	2.58
IL-4 (pg/ml)	7.75	4.19	6.06	1.32	2.1	2.58

In Day 10 (Table 6) mean levels of TNF-alpha were 60.87 ± 7.82 pg/ml, compared to IL-12 was found to be 47.5 ± 8.27 pg/ml and IL-4 were 7.75 ± 4.19 pg/ml.

In the control group the values of TNF-alpha, IL-12 and IL-4 were 42.9 ± 13.5 pg/ml, 49.8 ± 11.59 pg/ml, and 6.06 ± 1.32 pg/ml respectively.

Discussion

One of the major unresolved questions in malaria is why some patients with *Plasmodium falciparum* infection become so sick and die. Cell–cell interactions between the parasite and the host involving adherence/invasion appear generally, but not exclusively, to correlate with severity. The most important of these interactions in the asexual blood cycle are: (i) the invasion of red cells by merozoites, (ii) the binding of PRBC to uninfected red cells (rosetting), (iii) the binding of PRBC to endothelial cells in critical organs (cytoadherence) and (iv) the induction of proinflammatory cytokines by PRBC, notably tumour necrosis factor (TNF-α). The resulting clinical manifestations are protean. Analysis of these cellular interactions has revealed marked heterogeneity in molecular specificity which highlights the complexity of pathogenesis, but also opens the way to new modalities for treating this deadly infection [8].

The essential mechanism of death in falciparum malaria disease is agreed by many researchers: a functional tissue hypoxia that forces an unsustainable dependence on anaerobic metabolism. An unresolved key question is whether the tissue hypoxia arises (a) because insufficient oxygen reaches the mitochondria through either vascular occlusion from sequestered parasitized red cells acting alone, or in combination with anaemia or (b) because excessive release of inflammatory cytokines, induced by malarial toxin(s), render mitochondria unable to use oxygen to generate energy from oxidative phosphorylation.

Current basic literature suggests that inflammatory cytokines are very much the dominant partner, having amongst their powers the capacity to shut down bone marrow, make red cells prematurely poorly deformable, and channel sequestration towards certain sites, dictated by innate local thrombomodulin concentration. Thus cytokines and poor oxygen delivery should not be viewed as alternative theories of malarial disease pathophysiology. Instead, the latter is one of the consequences of the former [9].

In our study, TNF-alpha, IL-12 showed significant increase and IL-4 showed a persistent decline on Day 0 (Table 1) as expression of immune activation in response to the presence of parasites. Analyzing the results from Tables 1, 2, 3, 4, 5 and 6 we get an vital information, substantial elevation in TNF-alpha and IL-12 shows statistically significant decrease from Day 0 to Day 10, conversely IL-4 which dipped to a low in Day 0 shows statistically significant elevation as we approach Day 10, finally on Day 10 (Table 6) IL-12 and IL-4 reach the normal values but still TNF-alpha remains elevated. Further these observations from Day 0 to Day 10 show a direct correlation with the improved clinical condition of the 32 patients included in the study as shown in Table 7. A large body of evidences indicates that cytokines are determinants of malaria severity and outcome [5–8] and can represent potential targets for therapeutic interventions, if their effect will be highlighted.

Several studies suggest that the balance between pro-inflammatory cytokines such as TNF-alpha and anti-inflammatory cytokines such as IL-4 determines the degree of malaria parasitaemia, level of anaemia and clinical severity [10]. Other evidences suggest that malaria outcome depends on cytokine overproduction and not on the balance between them, since high levels of anti-inflammatory as well as pro-inflammatory cytokines may be associated with disease severity and mortality [11]. In human malaria altered immune reactivity appears late in the acute phase of the disease and can last a long time after the clearance of parasites from the circulation.

An explanation for the poor acquisition of malaria immunity in naturally exposed populations is that the parasite actively modulates the immune system of the host, preventing the development of specific immune responses [12].

Table 7 Clinical and parasitological measures in *P. falciparum* patients ($n = 32$) from Day 0 to Day 10

Clinical parameters	Day 0 ($n = 32$)	Day 2 ($n = 32$)	Day 4 ($n = 32$)	Day 6 ($n = 32$)	Day 8 ($n = 32$)	Day 10 ($n = 32$)
Hyperparasitemia >250,000 parasites/μl	30	26	20	17	10	02
Severe anemia Hb level <6.0 g/dl	30	24	19	12	09	01
Hemoglobin (g/dl)	6.1 ± 0.98	7.6 ± 1.64	8.1 ± 1.94	9.4 ± 1.43	10.1 ± 1.21	12.91 ± 1.92
Serum iron (mg/dl)	70.1 ± 0.73	81.1 ± 1.71	85.86 ± 1.86	96.6 ± 2.13	99.47 ± 3.12	118.92 ± 5.18
Temperature (°C)	41 ± 0.2	39.8 ± 0.2	39.2 ± 0.2	39.0 ± 0.2	38.4 ± 0.2	38 ± 0.2

The inflammatory response that is needed to remove parasites leads to considerable tissue damage and activation of phagocytes to kill intracellular or extracellular parasites requires the production of inflammatory cytokines, which can cause systemic effects such as severe anaemia and CM [13, 14]. The outcome of infection depends on a delicate balance between appropriate and inappropriate induction of these mediators.

High levels of TNF in particular are associated with fever, which closely follows schizogony, and TNF release by monocytes cultured with erythrocytes infected with *P. falciparum* in vitro coincides with schizont rupture [15]. A trial with a neutralizing monoclonal antibody against TNF on infected children in the Gambia showed a direct correlation between the dose of antibody given and the speed of return to a normal temperature [16].

TNF-α has a role in the regulation of macrophage interleukin 12 production, and it has been shown that TNF-α is an important co-factor for interleukin-12-induced production of interferon γ by NK cells [17]. Moreover Luty et al. [7] showed a close association between the presence of severe anaemia, high TNF-α concentrations, and large numbers of circulating haemozoin containing monocytes, suggesting that haemozoin-induced TNF-α production plays a part in either initiation or exacerbation of anaemia as a clinical outcome of chronic, uncontrolled parasitaemia.

Interleukin 12 seems to stimulate antibody production in B cells and it has been shown that interleukin 12 is effective in inducing protective immunity against blood-stage infection in the murine model [18]. It is likely that even the process of phagocytosis stimulates interleukin 12 production. Interleukin 12 acts on antigen-stimulated CD4+ T cells, activating signal transducers and activating transcription 4 (STAT 4) and promoting the differentiation of T cells into the Th1 subset. The Th1 effectors produce interferon γ, which acts on macrophages to stimulate their microbicidal functions and to increase their production of interleukin 12. The raised concentrations of interleukin 12 modulate macrophage activity, which is associated with increased erythrocyte destruction and bone marrow dyserythropoiesis [18].

It seems that early events in the cell-mediated immune response needed for defence against malaria, initiated by the release of interleukin 12 from monocytes/macrophages, B cells, and other cell types [7, 18] and consequently the concentration of interleukin 12, reveals a prognostic significance in malaria infection.

Evidence from recent results establishes a critical role for interleukin 12 in the adaptive immune response to malaria and confirms the association between levels of interleukin 12 and macrophage activation, with production of TNF-α, directly related to the effects of haemozoin on phagocytic cells. Evidence suggests that interleukin 12, produced by macrophages in response to infectious agents, is a central mediator of the cell-mediated immune response by its actions on the development, proliferation, and activities of Th1 cells [19]. In our study IL-12 levels were more than five times the normal values on Day 0 but statistically significant reduction started from Day 2 and by Day 10 (Table 6) the elevated IL-12 levels were restored to near normal. Most significantly the above changes closely corroborated with improvement in their clinical parameters as shown in Table 7.

IL-4 is a cytokine that participates in the regulation of the immune system at multiple levels [20, 21]. IL-4 is a growth and survival factor for lymphocytes [22–24]. Although it was discovered as a B cell differentiation and stimulatory factor [22] its role in regulating T cell differentiation is critical during the immune response [25, 26]. After antigen challenge, resting T cells differentiate towards Th1 or Th2 cells [26]. The mechanisms involved in this process are dependent on cytokines. IL-4 plays an essential role by promoting Th2 cell differentiation while inhibiting Th1 cell +differentiation [22]. IL-4 is also able to protect lymphoid cells from apoptosis [23, 24], but it is unable to promote proliferation of small resting lymphocytes without a co-stimulatory signal such as that provided through antigen receptor engagement [27]. The effects of IL-4 are not restricted to lymphoid cells. Thus, IL-4 can regulate proliferation, differentiation, and apoptosis in multiple cell types of haematopoietic and non-haematopoietic origin including myeloid, mast, dendritic, endothelial, muscular, and neuronal cells [28–30]. The pleiotropic effects of IL-4 reveals the important role that this cytokine plays during a normal immune response. IL-4 promotes Th2 cell responses through the IL-4Rα chain and STAT6 activation. This process is very important in a normal immune response as demonstrated during parasitic infection. As in case of TNF-alpha and IL-12 in the present study IL-4 levels dipped significantly lower than the normal values in Day 0 but during the course of the study it rose significantly and by Day 10 it was restored to normal. In IL-4 levels also the changes their values from Day 0 to Day 10 correlated to the statistically significant improvement (Table 7) in all clinical parameters of the 32 patients which were part of the study.

Our results indicating the levels of TNF-alpha, IL-12 and IL-4 from Day 0 to Day 10 reveals a prognostic significance in falciparum malaria infection. The intensity of elevation in TNF-alpha was more steeper compared to IL-12. We found a sequential persistent decrease in levels of TNF-alpha and IL-12 from Day 0 to Day 10 [955 ± 261.33 pg/ml (day 0) to 60.87 ± 7.82 pg/ml (Day 10) and 293.12 ± 94.64 pg/ml (Day 0) to 47.5 ± 8.27 pg/ml (Day 10) respectively] whereas similar sequential elevation in IL-4 levels from Day 0 to Day 10 [2.35 ± 0.64 pg/ml (day 0) to

7.75 ± 4.19 pg/ml (Day 10)] portraying crucial evidence associating sequential cytokine levels with prognosis in *P. falciparum* malaria. We further had an important observation that the changes in the cytokine levels from Day 0 to Day 10 was corroborated with clinical improvement in the patient's medical condition accompanied by anti-malarial treatment which is portrayed in Table 7. Further studies with larger sample size would add appreciably to our understanding of the role of cytokines in disease severity and prognosis associated with *P. falciparum* infection.

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