

TUMOR NECROSIS FACTOR IN FALCIPARUM MALARIA

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To investigate the relationship of TNF α levels to *Plasmodium falciparum* (PF) infection, plasma TNF α concentrations were measured in Pakistani adults and children with mild, severe, cerebral and chronic falciparum malaria and healthy (control) subjects. The initial geometric mean plasma concentrations of TNF α in adult patients with severe malaria (187.6 pg/mL) were significantly higher than mild malaria patients (87.1 pg/mL, $P < 0.001$). TNF α levels were not correlated to parasite density, cerebral malaria, young age, hypoglycemia or fatal outcome; however, they were associated with severe anemia, and hepatic and kidney dysfunction. TNF α levels were not significantly increased in 16 patients with hyperparasitemia and were significantly elevated ($P < 0.02$) in chronic malaria patients as compared to control subjects. TNF α levels were elevated independently in patients with anemia, hypoglycemia ($P < 0.001$, $P < 0.05$), and hepatic and kidney ($P < 0.001$ each) dysfunction. In this study, high TNF α levels were associated with several manifestations of severe malaria and were not specific to cerebral malaria and hyperparasitemia. *Ann Saudi Med* 1996;16(6):609-614.

Malaria attacks about 300 to 500 million people each year, mostly in the tropics, and causes 2.7 million deaths annually. Almost half of the world population is at risk from this disease. Falciparum malaria is responsible for almost all of the two million or more deaths attributed to malaria each year worldwide.¹ People who live in endemic areas and have been frequently infected acquire some immunity, so that they tolerate *Plasmodium falciparum* parasitemia with mild or no symptoms. However, in nonimmune people, falciparum infection always causes debilitating illness and must be regarded as a potentially fatal disease.²

In endemic areas, falciparum malaria is associated with a number of complications involving different organ systems, increased mortality and residual neurological deficit, especially in children.³ Again, in highly endemic areas of malaria, the clinical picture is variable. At one extreme, previously healthy individuals suffer from an acute attack. These patients usually have a high parasite count. At the other extreme, a group of patients present with a longer history of febrile illness. These patients have general symptoms of ill health, a low-grade fever,

are markedly anemic and have a low parasite count in their peripheral blood. They are labeled to be suffering from chronic falciparum malaria.

In Pakistan, the malaria risk exists throughout the year in the entire country, except in the high mountains in the north. From 1986 to the present, *Plasmodium falciparum* has emerged as the predominant species infecting the people.⁴ Resistance of the malarial parasite to chloroquine has been reported from many areas of Pakistan.⁵

Tumor necrosis factor (TNF α), a cytokine produced by monocytes, macrophages and T lymphocytes, is thought to play an important role in host response to inflammation and infection.⁶ In 1986, raised TNF α levels were detected in patients with malaria.⁷ Since then, interest has focused on the possible relation of TNF α to severe and cerebral malaria. There is now considerable evidence linking TNF α with various aspects of malarial pathology, including reports of raised TNF α levels in sera from malaria patients.⁸ It has also been reported that serum TNF α levels correlate with disease severity in malaria.⁹

Systematically, TNF α levels in falciparum malaria in Pakistan have not yet been reported. Studies were therefore carried out both in adults and children suffering from malaria and normal healthy controls. All the subjects studied lived permanently in malaria-endemic areas. The objectives of this study were 1) to measure TNF α in the plasma of *Plasmodium falciparum* infected subjects, 2) to correlate the presence of TNF α to symptomatology, parasite density and other biochemical parameters, 3) to compare the clinical and biological

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aspects of severe malaria in adults with severe malaria in children, and 4) to measure TNF α in the plasma of healthy subjects and to compare it with the values found in other countries.

Patients and Methods

Seventy-four Pakistani adults and 26 children who were admitted to Lady Reading Hospital, Peshawar, and District Headquarters Hospital, Dera Ismail Khan, between August 1994 and February 1995 with acute symptoms of malaria due to *Plasmodium falciparum* were selected for this study. Twenty-five healthy subjects were also included in the study as controls. The underwritten clinical groups were defined.

Mild Malaria (MM)

Febrile illness in a subject with asexual *Plasmodium falciparum* parasites in blood film, without any other satisfactory explanation for the fever, and without cerebral or severe malaria, was diagnosed as mild malaria.

Severe Malaria (SM)

Subjects that showed severe malaria due to *Plasmodium falciparum* hyperparasitemia (>100,000 parasites/ μ L) or any presence of *P. falciparum* parasitemia and one of the following: coma, hypoglycemia (glucose < 2.2 mmol/L), or severe anemia (hematocrit < 20% or hemoglobin < 7.0 g/dL), serum creatinine >3.0 mg/dL and serum transaminase level >100 units/L, were diagnosed with severe malaria.

Cerebral Malaria (CM)

Patients were considered to have cerebral malaria if they were comatose and had detectable *Plasmodium falciparum* asexual forms in peripheral blood and if there was no other recognized cause of alteration of consciousness.

Chronic Malaria (CHM)

Children with detectable parasites of *P. falciparum* asexual forms in peripheral blood, and with a prolonged duration of illness ranging from at least three months to one year and without any other satisfactory explanation for the fever, were considered to have chronic malaria.

Controls

Controls included 25 healthy subjects (adults and children). None of the controls was parasitemic at the time of sampling. All the control subjects belonged to the same malaria-endemic area.

Laboratory Investigations

Blood samples were taken within 24 hours of admission before the initiation of antimalarial treatment. Routine hematological investigations, i.e., total leukocyte count (TLC), hemoglobin level (Hb), red blood cell (RBC) count, erythrocyte sedimentation rate (ESR) and fibrinogen degradation products (FDP) were determined. Biochemical parameters like blood glucose, serum creatinine, serum bilirubin, alanine aminotransferase, lactate dehydrogenase and serum albumin were also measured. Platelet count was not done due to technical reasons. Parasitemia (the number of asexual *P. falciparum* parasitized erythrocytes/1000 red cells in a thin film or the number of parasites/200 WBC on thick film) was determined after staining with Giemsa stain.

TNF α Assay

For the determination of TNF α , venous blood samples were taken from the anterior cubital vein within 24 hours of admission of the patients and allowed to clot. The samples were centrifuged, sera were separated and stored at -20°C until cytokine assay. Serum concentrations of TNF α were assayed as described previously by Kwiatkowski,¹⁰ using an enzyme-linked immunosorbent assay.

TNF was measured using the quantitative immunoenzyme metric sandwich technique (Biotrack TNF α , human, ELISA, system, code, PRN 2148, Amersham, Life Science, England).

The assay system for TNF α is based on a solid phase ELISA, which utilizes a highly specific monoclonal antibody for TNF α bound to the wells of a microtiter plate, together with a polyclonal antibody to TNF α conjugated to horseradish peroxidase. TNF α present in a serum sample is bound by immobilized antibody. After washing away any unbound sample proteins, an enzyme-linked polyclonal antibody specific for TNF α is added to the wells and allowed to bind the TNF α , which was bound during the first incubation. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of TNF α bound in the initial step. The color development is stopped and intensity of the color is measured. The intensity of the color produced is proportional to the amount of TNF α in the sample. TNF α concentration in each sample was determined by direct interpolation of corrected absorbance values from the calibration curve.

The diluent used in the assay technique was animal serum. Since the diluent is not different from the serum samples, it will not affect the TNF α level. The assay technique used is highly sensitive and specific. The sensitivity of this assay technique with animal serum as

standard diluent is 4.4 pg/mL. This technique recognizes both natural human TNF α and recombinant human TNF α . No significant cross-reactivity or interference is observed.

Statistical Analysis

Statistical analysis of clinical and laboratory data were carried out using a statgraphic program (version 5.0). Normally distributed data were analyzed using Student's *t*-test (all *P* values are two-tailed). The Wilcoxon's signed rank tests and the Kruskal Wallis one-way Anova were used for abnormally distributed data. Pearson's correlation coefficient was used to evaluate the relationship within normally distributed variables, and the Spearman rank correlation for the remainder.

Results

From August 1994 to February 1995, during a seven-month period, 1575 slides were examined for the detection of malarial parasites. The positivity rate of the slides was 48%. Of the positive slides, 98.7% showed *P. falciparum*, while 1.3% showed *P. vivax* parasites. Overall, 100 patients (74 adults and 26 children) were enrolled.

Age of the Patients

The mean age in severe malaria in adults (SMA) was 38.8 years (range 16-50); in MM in adults (MMA) it was 31.4 years (16-70); in severe malaria in children (SMC), 5.09 years (0.9-12); in MM in children (MMC), 8.2 years (4-14) and in CM it was 32.2 years (14-50).

Admission Clinical and Laboratory Variables

Splenomegaly was present in 64% of the patients, hepatomegaly in 29%, hepatosplenomegaly in 21%, jaundice in 18%, severe anemia (Hb < 7.0 g/dL) in 10.8%, hepatic dysfunction (ALT >100 U/L) in 8.0%, kidney dysfunction (creatinine >3.0 mg/dL) and multiple organ dysfunction in 4%.

The mean duration of symptoms before presentation in SMA was 40.2 days (range 3-365), in MMA, 34.05 days (2-365), and in SMC, 23.06 days (4-365). The mean duration of symptoms as a whole was more, since in 12 patients the duration of symptoms was from three months to one year.

TNF α Concentration

The geometric mean TNF α concentration for adults in SM (n=32) was 187.68 pg/mL (median 155, range 62-2000). One adult had an extremely high TNF α level, i.e., 2000 pg/mL. This adult male (age 22 years) presented with a lower motor neuron-type lesion. The children in the SM group (n=11) had a geometric mean TNF α level of 198.37 pg/mL (median 240, range 30-950), the adults and children in the MM group had a geometric mean TNF α

level of 87.1 pg/mL (median 112.5, range 5-500), and 80.4 pg/mL (median 80, range 5-125) respectively. In the CM group (n=11), excluding other forms of the SM group, all the patients were adults. They had a geometric mean TNF α concentration of 134.9 pg/mL (median 120, range 20-520). One child and two adults in the MM group had undetectable TNF concentration. For statistical purposes, they were assigned the values of 5 pg/mL each.

The control group (n=25) had a geometric mean TNF α level of 97.3 pg/mL (median 110, range 5-145). One subject in the control group had an undetectable TNF α level. The patients in the chronic malaria (CHM) group (n=12) had a prolonged duration of illness ranging from three months to one year; the geometric mean TNF α level was 148.5 pg/mL (median 112.5, range 30-950).

Patients in the SMA (severe malaria, adults) group had significantly higher (*P* < 0.001) TNF α levels compared with patients in the MMA (mild malaria, adults) group. Although the geometric mean TNF α level in children with severe malaria (SMC) was more than in children with mild malaria (MMC), the difference was statistically insignificant. Similarly, the geometric mean TNF α level in the CMA group was more than in the MMA group, but the difference was statistically insignificant. Sixteen patients in our series had hyperparasitemia, i.e., the parasite count was >100,000 μ L of blood. The geometric mean TNF α level in this group was 161.1 pg/mL (median 54, range 52-97). The difference between the high parasitemic group and all other patients (n=84) with a low parasite count was statistically insignificant.

When compared with the control group, the SMA and SMC groups had a statistically significant difference (*P* < 0.001, *P* < 0.01 each) of TNF level. The mild malaria group TNF levels (both adults and children) did not differ significantly from the control group except with the chronic malaria group, in which the difference was statistically significant (*P* < 0.02). Three patients who died had TNF levels of 450, 250 and 20 pg/mL respectively. Although the levels were lower than the SMA group, the difference was statistically insignificant from the survivors in the same group.

Parasitemia

Levels of parasitemia at the time of the blood sample collection ranged from 2500 to 558,000 parasites/ μ L. The geometric mean parasite count and TNF α levels in various groups of malaria are shown in Table 1. The difference between the parasite count in the SMA group and the MMA group was highly significant (*P* < 0.001). Although the geometric mean parasite count in severe malaria in children (13,701.1 parasites/ μ L) was more as compared to mild malaria in children (4649.06 parasites/ μ L), the difference was statistically insignificant. The difference between the parasite count in the CM group in adults and

the MMA group was also statistically significant ($P < 0.02$). Although the geometric mean parasite count in severe malaria in adults was more, compared to severe malaria in children, the difference was statistically insignificant.

Other Hematological and Biochemical Parameters

The mean \pm SD values of various hematological and biochemical parameters are shown in Tables 2 and 3. The mean values of hemoglobin, blood sugar, serum creatinine, serum bilirubin, alanine aminotransferase and lactate dehydrogenase were higher in patients from the SMA group compared to the MMA group, the difference shown to be statistically significant ($P < 0.001$, $P < 0.02$, $P < 0.05$, $P < 0.02$, $P < 0.025$, $P < 0.001$) for each by Student's *t*-test. The difference in the hemoglobin level, serum creatinine and lactate dehydrogenase between the CM group in adults and the MM group was statistically significant ($P < 0.02$, $P < 0.02$, $P < 0.025$), shown by Student's *t*-test. The serum albumin and fibrinogen degradation products (FDP) level in the SM group and the CM group did not differ significantly from the MM group (both adults and children in either case).

TABLE 1. *TNF α levels and parasite counts.*

Groups	No. of patients	TNF *pg/mL	Parasite count per ** μ L
SMA	32	187.68	23,143.8
SMC	11	198.37	13,701.1
MMA	40	87.1	5884.7
MMC	15	80.4	4694.06
CMA	11	134.9	12,093.5
CHM	12	148.5	5211.6
HPC	16	161.4	203,260.0
Control	25	97.3	—

SMA=severe malaria adults; CMA=cerebral malaria adults; SMC=severe malaria children; MMA=mild malaria adults; MMC=mild malaria children; CHM=chronic malaria; HPC=high parasite count; *=geometric mean values; **=geometric mean.

TABLE 2. *Hematological laboratory parameters.*

Groups	Hb (gm/dL)	Mean \pm SD	FDP (μ g/mL)	Mean \pm SE
SMA	10.5 \pm 3.2	n=27	15.5 \pm 3.8	n=27
SMC	9.3 \pm 0.7	n=11	10.2 \pm 3.7	n=10
MMA	12.1 \pm 1.4	n=38	17.7 \pm 2.9	n=39
MMC	10.8 \pm 2.1	n=14	13.6 \pm 3.9	n=14
CHM	9.7 \pm 3.5	n=12	—	—
HPC	11.0 \pm 2.4	n=14	26.1 \pm 7.3	n=12

TABLE 3. *Biochemical laboratory parameters.*

Association Between Plasma Factors and the Severity of the Disease

In the SMA group, as well as the SMC group, TNF α levels did not increase with increasing *P. falciparum* parasite densities ($r=0.25$, $P=0.25$). In the CM group, TNF α concentration increased with increasing age; however, in the SM group (both adults and children), TNF α levels did not correlate with the age. Blood glucose concentrations in the SMA group, as well as the SMC group and the CM group in adults, were not significantly associated with the TNF α levels ($r=0.3$, $P=0.25$). Hemoglobin levels in the SM group (both adults and children) and the CM group correlated significantly with the TNF α levels ($r=0.5$, $P=0.05$, $r=0.06$, $P=0.05$ respectively for each) by Spearman rank correlation.

In SMC and CM in adults, the TNF α levels correlated directly with increasing serum creatinine levels ($r=0.9$, $P=0.001$, $r=0.4$, $P=0.01$), each by Spearman rank correlation. No significant correlation was found between TNF α levels and serum creatinine levels in the SMA group. TNF α concentrations in the SMA and SMC groups correlated significantly with serum bilirubin levels ($r=0.7$, $P=0.001$, $r=0.89$, $P=0.001$); however, this correlation was not found in the CM group in adults. Significant correlation was found between TNF α levels and lactate dehydrogenase levels in the SMA group only ($r=0.3$, $P=0.05$).

Three patients expired during our study. All three were suffering from cerebral malaria. The overall mortality rate was 3%. In the CM group, the mortality rate was 27%. TNF α levels in two patients in this group were high (450 pg/mL and 250 pg/mL), while it was very low in the third patient (20 pg/mL). There was no significant difference in TNF α levels in survivors and nonsurvivors.

Discussion

Falciparum malaria is a multifactorial clinical syndrome and as such, is a disease of protean clinical manifestations. Data from several recent studies have suggested an association between the *P. falciparum* infection and elevated TNF α levels.¹¹ Most of the previous studies were carried out in children. To the best of our knowledge, only two studies have been undertaken in adults.^{12,13}

Our study differs from the previous reports, as we compared TNF α levels in children and adults suffering from falciparum malaria, as well as control subjects, all from the same malaria-endemic area. This study provides evidence that TNF α production is increased in *P. falciparum* infection. In addition, our findings

Groups	Glucose mg/dL Mean ± SE	Creatinine mg/dL Mean ± SE	Bilirubin mg/dL Mean ± SE	Alt U/L Mean ±SE	LDH U/L Mean SE	Albumin gm/dL Mean ± SD
SMA	78.3±6.0 n=29	1.4±0.2 n=32	3.2±0.98 n=32	63.8±12.3 n=32	556.5±72.4 n=32	4.4±0.1 n=26
SMC	50.9±8.5 n=8	0.9±0.1 n=11	3.6±1.4 n=11	110.9±44.2 n=11	560.7±65.9 n=10	4.6±0.2 n=8
MMA	96.3±5.0 n=36	0.9±0.05 n=38	1.2±0.2 n=40	37.1±4.06 n=40	326.5±43.6 n=38	4.3±0.1 n=29
MMC	77.4±10.5 n=8	0.9±0.1 n=13	0.7±0.1 n=14	30.4±5.6 n=14	728.8±158.04 n=3	4.8±0.1 n=7
CHM	69.8±8.7 n=11	—	1.2±0.2 n=12	33.8±7.3 n=12	389.3±75.9 n=2	—
HPC	83.2±9.9 n=14	1.7±0.4 n=16	6.0±1.9 n=6	118.3±32.7 n=16	543.1±66.7 n=15	4.3±0.1 n=15
Control	97.9±4.2 n=25	—	—	—	158.2±13.2 n=25	5.4±0.08 n=25

suggest that elevated TNF α levels can be associated with pathophysiological features of severe malaria, particularly jaundice, hepatic and kidney dysfunction. Further, it provides evidence that increased TNF α levels are not specific to cerebral malaria infection, as reported in other studies.¹⁴ Many workers have demonstrated that TNF α levels correlate significantly with the parasite density;^{11,15} however, we did not find such correlation in our hyperparasitemic patients.

Sixteen patients in our study had hyperparasitemia. However, only 25% showed evidence of one- or two-organ dysfunctions. These patients did not have significant elevation of TNF α levels. This suggests that our population has some degree of immunity. This may operate to prevent illness through the presence of an antibody to TNF-inducing antigens that have been shown to be present in falciparum malaria,¹⁶ thereby reducing macrophage activation and TNF output.¹⁷ Another important factor may be the stage of parasite development found in the blood in these patients. In all these patients, mostly tiny rings and young trophozoites of *P. falciparum* were seen in the blood film. Silamut and White demonstrated that with any parasitemia, the prognosis is worse if the parasites are mature.¹⁷

TNF α levels correlated significantly with the disease severity as reported by other workers.¹⁹ Although TNF α levels were elevated in patients with hypoglycemia and cerebral malaria, this association was not constant or significant. Although some workers had demonstrated the association of TNF α levels with cerebral malaria,^{9,14} our study did not show a significant correlation. Some of our findings duplicate the reports of Shaffer et al.¹¹ in demonstrating the association of significantly elevated TNF α levels with severe anemia, which has been reported by very few workers.

Another interesting observation made during this study was organ impairment (liver and kidney dysfunction) in falciparum malaria. This organ dysfunction was found to be significantly ($P < 0.001$) associated with increased circulating levels of TNF α . A similar association had been reported earlier by Kern et al.¹²

Most of the researchers are of the opinion that falciparum malaria produces more severe disease in children, probably due to lack of immunity. However, we did not find any significant difference between the severe criteria and most of the biological features (glycemia, parasitemia, hemoglobin levels, liver function tests, kidney function tests, FDP, LDH, and serum albumin levels) of severe malaria in adults and children. Similar findings have also been reported by Saissy et al. in a seasonal malaria-endemic area in West Africa.²⁰ Although their sample size was small, they found that serum levels of IgM and IgG were significantly increased in the adult group.

We were unable to demonstrate the relationship between TNF α levels with fatal outcome. This may be explained in part by a lower proportion of patients who died, or by differences in the time between determination of TNF α levels and death. The degree to which high TNF α levels contribute to mortality, or simply reflect premorbid processes, needs to be clarified. It is also clear from our work that 6% of our patients had significantly high levels of TNF α (range 200 to 500), but they recovered rapidly with appropriate treatment. Since malaria is endemic in Pakistan, these patients may have developed clinical immunity due to the development of antibodies against the exoantigens they have encountered during the course of frequent infections, as demonstrated by Bate et al.²¹

Recently, it has been shown that glycosyl phosphatidyl inositol (GPI) is a novel class of glycolipid toxins produced

by the parasite. GPI stimulates high levels of TNF α and interleukin 1 production by macrophages. These mediators are responsible for malarial pathophysiology.²² It is still not established what part TNF α plays in malaria, but the possible role of anti-TNF α antibodies in cerebral malaria is under investigation and it was reported in 1993 that monoclonal antibodies against TNF α reduce fever in children with cerebral malaria.²³ Kumaratilake et al. synthesized TNF α agonist peptide-TNF (70-80) and demonstrated that it enhanced the polymorphonuclear (PMN) mediated killing of *P. falciparum* *in vitro* and reduced the *P. chabaudi* parasitemia in mice. They concluded that host-protective effects of TNF can be retained, while the toxic effects are eliminated using a selected characterized subunit of the cytokine.²⁴

In conclusion, our findings established that TNF α levels are elevated in patients with *Falciparum* malaria. Our data provide further confirmation of the clinical association between elevated TNF α levels and several different manifestations of severe *P. falciparum* infection, particularly anemia, hepatic and kidney dysfunction. This study shows that high TNF α levels are not apparently specific to cerebral malaria. We propose from our findings that clinical immunity may play a protective role in hyperparasitemic patients with acute *P. falciparum* malaria. This work provides information that in malaria-endemic areas, adults and children with severe malaria do not differ in clinical or biological aspects. The presence of relatively elevated TNF α levels in healthy control subjects in our situation may play a role in clinical immunity.

It is proposed to study the immunological profile in our falciparum malaria patients on a large scale and the genetic makeup of the concerned malarial parasite, as TNF production is likely to be determined by at least four factors, i.e., strain variation in toxin production by the parasite, variation in the propensity of the host to produce TNF, the population dynamics of the parasite within the host, and the acquisition of antitoxic antibodies and other immune adaptations of the host.²⁵

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