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INCREASED TUMOR NECROSIS FACTOR ALPHA AND INTERLEUKIN-6 SERUM LEVELS AND THEIR CORRELATION WITH LABORATORY PARAMETERS IN PATIENTS WITH IMPORTED MALARIA

JASMINA POLUGA[1,2], VIOLETA DOPSAJ[3,4], MILENA VELJKOVIĆ[5], N. MAKSIĆ[4], SONJA STOJAKOVIĆ[4], RADICA DUNJIĆ[4], ZORICA DAKIĆ[6] and MILORAD PAVLOVIĆ[1,2]

[1] *Department of Infectious and Tropical Diseases, University of Belgrade, Faculty of Medicine,* 11000 Belgrade, Serbia
[2] *Clinic of Infectious and Tropical Diseases, Clinical Center of Serbia,* 11000 Belgrade, Serbia
[3] *University of Belgrade, Faculty of Pharmacy,* 11000 Belgrade, Serbia
[4] *Institute of Medical Biochemistry, Clinical Center of Serbia,* 11000 Belgrade, Serbia
[5] *Sveti Sava Hospital,* 11000 Belgrade, Serbia
[6] *Parasitological Laboratory, Department of Microbiology, Clinical Center of Serbia,* 11000 Belgrade, Serbia

Abstract - In malaria, blood concentrations of proinflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) and interleukin (IL)-6, are increased. In a study which included 34 patients, TNF-α and IL-6 were examined in two phases, immediately after the admission of patients, and at the end of antimalarial therapy, when the parasitemia was negative. The results show a significant increase of TNF-α and IL-6 in the first phase, before the effects of antimalarial therapy. A very strong correlation between TNF-α and IL-6 is also confirmed, which suggests their coordinated production. Increased TNF-α values were correlated with an older age, the level of parasitemia, the number of platelets and leukocytes, elevated values of procalcitonin, D-dimer and lactate dehydrogenase, and lower values of serum iron and antithrombin. Increased values of IL-6 were correlated with the level of parasitemia, the number of platelets and leukocytes, and elevated values of D-dimer and lactates.

Key words: Malaria, cytokines, TNF-α, IL-6, parasitemia

INTRODUCTION

Patients with malaria, especially in severe forms, have an increased concentration of proinflammatory cytokines, of which TNF-α has been the most often examined (Chen et al., 2000). The increasing concentration of TNF-α in malaria was first recorded in 1986 (Scuderi et al., 1986). Kwiatkowski et al. (1989) demonstrated a significant increase in TNF-α levels in children with malaria in Gambia. In malaria, as well as in other systemic infections and diseases, the release of proinflammatory cytokines produces a systemic inflammatory response. Previous research related to sepsis and malaria has shown that TNF-α, the prototype of proinflammatory cytokines, increased in both diseases, causing hyperlactatemia. It is well known that increased regulation of proinflammatory cytokines in the process of adhesion to endothelial cells attracts circulating blood elements on the inner wall of the vessel (Sarangi et al., 2011). In many diseases, including malaria, this includes activated leukocytes and platelets, which play an important role in procoagulant activity. It is thought that proinflammatory cytokines increase the expression of molecules on the endothelial cells adhered to by parasitizing

erythrocytes. Proinflammatory cytokines enhance the expression of tissue factor on the endothelial cells and monocytes, and by generating the occurrence of thrombin, a molecule with significant roles in cross-reactions of inflammation and coagulation (Clark et al., 2006). The speed, time and intensity of release of cytokines varies considerably in different stages of the disease and between different individuals, thus forming a characteristic clinical feature. It is believed that proinflammatory cytokines are responsible for the occurrence of fever, nausea, vomiting, diarrhea, anorexia, myalgia, thrombocytopenia, immunosuppression, coagulopathy and neurological manifestations (Jennings et al., 2006). In severe forms of malaria, the concentrations of proinflammatory cytokines, such as TNF-α, IL-1, IL-6, and IL-8, are elevated in the serum. The high level of TNF-α in patients with falciparum malaria correlates with the severity of the disease, with the appearance of hypoglycemia, hyperparasitemia, jaundice, kidney damage, cardiovascular complications and death (Mordmüller et al., 2007).

The aim of this study was to determine the serum levels of TNF-α and interleukin-6, and to establish their correlation with other clinical and laboratory parameters.

MATERIALS AND METHODS

The study included 34 patients with imported malaria who were treated from 2007 to 2010 at the Clinic of Infectious and Tropical Diseases, Clinical Center of Serbia. From the total number of patients, 27 (79.41%) had falciparum malaria, while only 7 (20.59%) had malaria caused by the other type of plasmodium (vivax ovale).

The following data sources were used in this study: medical records, hematological, biochemical, parasitological and immunological analysis.

The most important parasitological analysis used in the study was the examination of thick and thin peripheral blood smears, stained with Giemsa. The level of parasitemia may be expressed either as a percentage of parasitized erythrocytes or as the number of parasites per microliter of blood.

The immunological analyses of the cytokines (TNF-α and IL-6) were performed at the Center for Medical Biochemistry, Clinical Center of Serbia, using the method of Quantikine ELISA Kit (R&D Systems).

The serum samples of patients for cytokine analysis were taken in two stages: immediately after the admission and at the end of antimalarial therapy, when the parasitemia was negative. After centrifugation, they were separated and stored at -70˚C until use.

The method by which the cytokines were determined was the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for TNF-α/IL-6 was pre-coated onto a microplate. Standards and samples are pipetted into the wells and any TNF-α/IL-6 present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for TNF-α/IL-6 was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and color developed in proportion to the amount of TNF-α/IL-6 bound in the initial step. The color development was stopped and the intensity of the color was measured.

The sensitivity (i.e. minimum detectable dose) of TNF-α was 1.6 pg/ml, and for IL-6, 0.70 pg /ml.

The following methods were used in statistical analysis: methods of descriptive statistics: mean value, standard deviation; Spearman's rank correlation to determine the correlation between the levels of cytokines and the other clinical and laboratory parameters; analysis of variance (ANOVA) to compare the levels of TNF-α in patients with malaria in relation to the control values; Kolmogorov-Smirnov test and Mann-Whitney test were used to compare the levels of IL-6 in patients with malaria in relation to the control values.

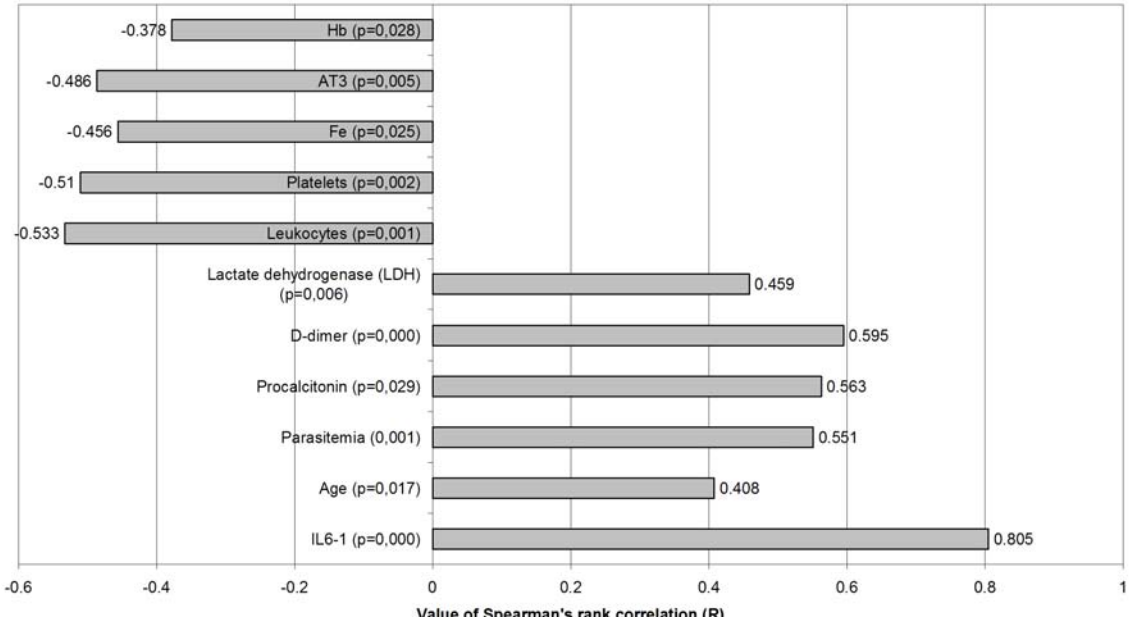

Fig. 1. Correlation on TNF-a with IL-6, the age and laboratory parameters in the first phase (1)

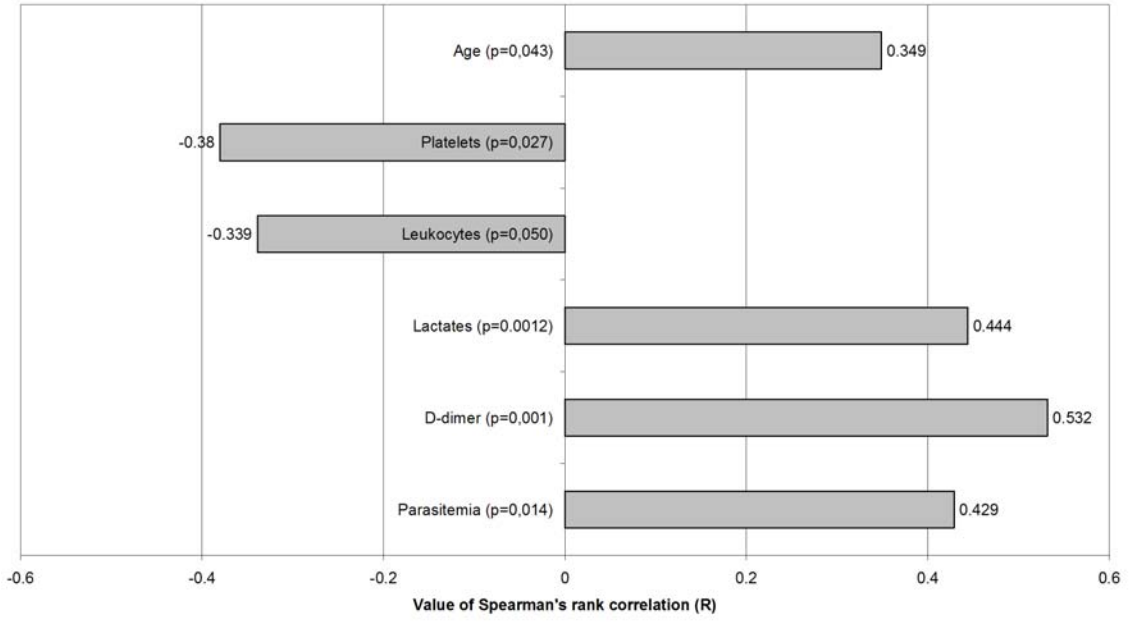

Fig. 2. Correlation on IL-6 with TNF-a, the age and laboratory parameters in the first phase (1)

RESULTS

The study included 34 patients. The cytokines TNF-α and IL-6 were examined at two phases: immediately after the admission of patients (1) and at the end of antimalarial therapy, when the parasitemia was negative (2).

Using the methods of statistical analysis (ANOVA, Kolmogorov-Smirnov and Mann-Whitney test) we have proved the increased values of TNF-α ($p=0.027$) and IL-6 ($p=0.006$) in the first phase.

The ANOVA test confirmed that there was no statistically significant difference in the values of TNF-α ($p=0.974$) and IL-6 ($p=0.827$) between the two phases, i.e. before and after treatment.

Additional laboratory parameters were also taken in each phase to determine the correlation and for monitoring dynamics in relation to the values of cytokines.

The values of D-dimer and C-reactive protein (CRP) were increased in the first phase, while the values of antithrombin III (AT3) and lactates were in the reference range.

In the second phase, there was a decrease in the value of laboratory parameters of the first phase, D-dimer and CRP. The values of AT3 and lactates were increased, but within the reference range.

The results show that there is a very strong correlation between TNF and IL-6 in the first phase. Increased TNF-α levels correlated with an older age, the level of parasitemia, as did the elevated values of procalcitonin, D-dimer and LDH, but also with the lower values of platelets, leukocytes, serum iron, AT3 and Hb.

The increased values of IL-6 correlated with the level of parasitemia, elevated values of D-dimer and lactates. A negative correlation was observed for thrombocytopenia and leucopenia. Unlike the TNF-α, elevated values of IL-6 poorly correlated with age.

The values of TNF-α in the second phase strongly correlated with the number of platelets. The values of IL-6 in the second phase strongly correlated with the value of D-dimer. It was concluded that in the first phase, before starting treatment, TNF-α and IL-6 correlated more with the laboratory parameters of the same phase.

DISCUSSION

The role of cytokines in the pathogenesis of malaria has been clearly confirmed, but not completely understood. It is thought that many factors impact on their level, which in the course of malaria depends on the circadian rhythm, and in accordance with this, there are large variations in the serum cytokine levels. TNF-α appears to be the most important in the early immune response and subsequent pathological manifestations. It has been proved that its concentration regulates the levels of the other cytokines as well, particularly in relation to IL-6 (Angulo and Fresno, 2002). Thus we could explain the results of this study, where the levels of TNF-α correlated to a high degree with other laboratory parameters in relation to IL-6, especially when taking into account the parameters that indicate the more difficult forms of disease (anemia, thrombocytopenia, high value of LDH). The results show a significant increase in TNF-α and IL-6 in the first phase, in the presence of parasitemia, and before the effects of antimalarial therapy. In addition, there is a very strong correlation between the levels of TNF-α and IL-6 in the first phase and their significant association with the level of parasitemia. Many other authors have also established their mutual coordination and association with the degree of parasitemia in patients with malaria (Shaffer et al., 1991; McGuire et al., 1998; Zeyrek et al., 2006).

According to a study from Hamburg (Kern et al., 1989), an increase in TNF-α and IL-6 was observed mainly in patients with falciparum malaria, while the concentrations of TNF-α and IL-6 correlated with the degree of parasitemia and the occurrence of complications. Thereby, they found a correlation between increased levels of these cytokines and anemia, hy-

Table 1. The values of cytokines in the first (1) and second (2) phase

Type of cytokines	Mean value (pg/ml)	SD
TNF-α (1)	21.03	17.39
IL-6 (1)	31.70	39.25
TNF-α (2)	5.17	3.59
IL-6 (2)	7.46	6.32

Table 2. The values of cytokines of the controls

Type of cytokines	Mean value (pg/ml)	SD
TNF-α	5.73	2.38
IL-6	3.92	2.19

Table 3. The values of additional laboratory parameters (phase 1)

Laboratory parameters	Mean value	SD
D-dimer	765.36	917.01
Antithrombin III (AT3)	85.55	18.01
Lactates	1.33	0.61
C-reactive protein (CRP)	114.41	84.88

Table 4. The values of additional laboratory parameters (phase 2)

Laboratory parameters	Mean value	SD
D-dimer	211.40	142.16
Antithrombin III (AT3)	100.32	13.76
Lactates	1.52	0.69
C-reactive protein (CRP)	49.43	116.03

Table 5. Correlation of TNF-α and IL-6 in the second phase (2)

Type of cytokines	Laboratory parameters	R	p
TNF-α (2)	Platelets (2)	(**) -0.472	0,015
IL -6 (2)	D-dimer (2)	(**) 0.531	0.006

poglycemia, hyperbilirubinemia and acute renal failure, which is consistent with the results of this study. A study from Pakistan showed almost identical results in falciparum malaria, but there was no correlation with the degree of parasitemia (Gandapup and Malik, 1996). Research conducted in Mali, in patients with falciparum malaria, showed a significant increase in cytokines in patients, including TNF-α and IL-6, but the correlation between hyperparasitemia and anemia with increased concentrations of IL-6 has not been proven (Lyke et al., 2004).

In the enlisted patients, increased levels of cytokines were in a stronger (TNF-α) or weaker (IL-6) correlation with the age. Authors from Pakistan (Gandapup and Malik, 1996) indicated that

the concentrations of TNF-α in children with falciparum malaria increased with age, whereas in adults there was no difference. Studies show that the concentrations of inflammatory mediators in serum/plasma, such as cytokines and acute phase proteins, increase with aging. The levels of circulating cytokines TNF-α and IL-6 are associated with morbidity and mortality in old age, and considered to cause or to exacerbate the risk factors in pathological conditions related to the age (Bruunsgaard et al., 2001; Krabbe et al., 2004). It is thought that in a relatively healthy elderly population, increasing levels of circulating IL-6 present a systemic response to local proinflammatory activity; however, when inflammatory diseases associated with old age progress, the level of circulating TNF-α increases, which gradually becomes a stronger marker of morbidity than IL-6 (Brüünsgaard and Pedersen, 2003).

The increased levels of TNF-α and IL-6 correlate with thrombocytopenia and a high concentration of D-dimer in both phases, i.e. before and after treatment, which is consistent with the pathophysiological mechanisms that occur in the course of this disease. In fact, the activation of the coagulation system leads to the appearance of fibrin degradation products (FDP), i.e. D-dimer in the circulation, which we found in malaria as well, mainly in those caused by *Plasmodium falciparum*. Initiation of the coagulation cascade and fibrinolytic system is caused by parasitized erythrocytes and/or activated platelets (Francischetti, 2008; Ghosh and Shetty, 2008). Parasitized erythrocytes adhere to the capillary endothelium, causing further activation which results in platelet consumption and the activation of coagulation, which may culminate in the appearance of DIC (disseminated intravascular coagulation). IL-6 stimulates platelets, which are manifested by an increased activation of thrombin and increased procoagulant activity, and coagulation markers detect changes as a response to the effects of TNF-α (Esmon et al., 1999). In the first phase, we found strong correlation between TNF-α and AT3, whose values are often reduced in falciparum malaria, especially its severe forms.

These results are expected when we have in mind the pathophysiological mechanism in which cytokine production and parasitized erythrocytes cause the consumption of AT3 (Mohanty et al., 1997). In contrast to the correlation of TNF-α and AT3, in the first phase we registered a correlation between increased levels of IL-6 and the increase of lactates. Some authors also find an association between increased concentrations of IL-6 and IL-10 and lactates, before starting antimalarial therapy (Day et al., 1999).

Sarthou et al. (1997) have shown a decline in the concentrations of TNF-α and IL-6 after antimalarial treatment. According to the results of this study, the average values of TNF-α and IL-6 in the second phase were much lower than values before therapy, but the difference in values was not statistically significant. The time interval between the determination of cytokine concentrations before and after treatment, i.e. during the presence or absence of parasitemia, was an average of 7 days, but for some patients this period was shorter. Specifically, these were patients who were taking antimalarial therapy on their own initiative, and the time of treatment was shorter. Most probably, this is the reason for the observed disparities between the levels of cytokines before and after treatment. Namely, after the therapy, they were generally reduced in all patients, albeit not equally.

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