Determination of the expression of lymphocyte surface markers and cytokine levels in a mouse model of *Plasmodium berghei* *

Funda Dogruman-Al1, Isil Fidan1, Semra Kustimur1, Kemal Ceber2, Turgut Imir1

1Gazi University Faculty of Medicine, Department of Medical Microbiology, Besevler, Ankara, Turkey; 2Nigde Government Hospital, Microbiology Laboratory, Nigde, Turkey

**INTRODUCTION**

Out of the 350-550 million malaria cases estimated to occur in the world every year, only around 1-2% are severe or life-threatening (Carneiro *et al.*, 2005). However, this small proportion represents an enormous malaria death toll per year, especially in sub-Saharan Africa, where more than 90% of the malaria deaths are thought to take place every year, affecting mainly children and pregnant women (Snow *et al.*, 2005). *Plasmodium berghei* (P. berghei) ANKA murine malaria has many features in common with human disease and is thus the best available model for certain important aspects of clinical malaria (Shofield and Grau, 2005). A detailed analysis of the mechanisms of protective immunity, its broad specificity and regulation as well as a potential role in pathology is most readily approached by the use of experimental animal models. *P. berghei* ANKA is uniformly lethal (via cerebral involvement) in all strains of mice infected (Langhorne *et al.*, 2002). Malaria disease manifestations vary with age and the acquisition of immunity, genetic polymorphism of host and parasite and regional variations and these manifestations appear to be regulated by the same factors (Eckwalanga *et al.*, 1994).

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**SUMMARY**

This study aimed to determine the changes in lymphocyte surface markers and cytokine profiles during a malarial infection in a mouse model of malaria. Mononuclear cells obtained from the spleens of the mice infected with *Plasmodium berghei* (P. berghei) were stained with anti-mouse CD3, anti-mouse CD4, anti-mouse CD8, anti-mouse CD19, anti-mouse CD152, anti-mouse pan natural killer (NK), anti-mouse CD80 monoclonal antibodies and expression of surface markers was evaluated by flow cytometry. In the serum samples of the mice, the levels of tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), transforming growth factor-β (TGF-1β), and interleukin (IL)-4, IL-10, and IL-12 cytokines were determined by ELISA method. The expressions of all the surface markers of lymphocyte evaluated were statistically significantly lower in the infected mice than in the healthy control mice (p<0.05). However, except for the level of TGF-1β, the levels of all the other cytokines evaluated were statistically significantly higher in the infected group than in the control group (p<0.001). No significant differences were determined between the TGF-1β levels of the study and control groups (p>0.05). In this study, T, B, and NK lymphocyte responses were inhibited and cytokine profiles changed in the course of malarial infection. Thus, interventions to increase the Th1 lymphocyte response may be beneficial in the prevention of malarial infection.

**KEY WORDS:** *Plasmodium berghei*, cytokine, lymphocyte surface markers, pathogenesis
The immune system is important for host defense. Host defense reactions against microorganisms are numerous and varied. The protective mechanisms against malaria are complex and not yet fully understood. Several studies have been carried out to elucidate the functional contribution of T cells to defense mechanisms against malaria (Troye-Blomberg et al., 1994). CD4+ T cells, which are essential for control of primary parasitemia in mouse models, produce pro-inflammatory cytokines that may participate in parasite clearance by triggering cellular immune responses (Langhorne et al., 2002). Cytokines are polypeptides produced by various cells such as mast cells, T and B cells, macrophages, endothelial cells. They regulate inflammatory and immune reactions (Abbas and Lichtman 2003). The T helper 1 (Th1) subset of CD4+ T cells secretes interferon-gamma (IFN-γ), Interleukin-2 (IL-2), tumor necrosis factor-1beta (TNF-1β) cytokines and is responsible for the activation of cell-mediated immunity and cytotoxic CD8+ T cells. Th2 subset of CD4+ T cells secretes IL-4, IL-6, IL-10 cytokines and is responsible for the stimulation of humoral immune response through which it helps B cell activation (Svirshchevskaya et al., 2001). Variations in human cytokine responses and their link to malaria disease manifestations are the subject of much debate (Eckwalanga et al., 1994). Inflammatory cytokines play an important role in human immune responses to malaria disease, although the pathogenic effects that can result from dysregulation and the balance between proinflammatory[(tumor necrosis factor-alpha (TNF-α), IFN-γ, IL-12) and anti-inflammatory cytokines [(IL-4, IL-6, transforming growth factor-1 beta (TGF-1β)] are poorly understood (Artavanis-Tsakonas et al., 2003). This study aimed to determine the changes in the lymphocyte surface markers and cytokine profiles during a malarial infection in a mouse model of malaria and thereby clarify the immunological mechanisms formed in malaria infection.

MATERIALS AND METHODS

In this study, 11 male Swiss Albino mice with an age range of 14-16 weeks (weight range: 25-30 gr) were used. The control group consisted of 15 male mice with the same characteristics. The mice were infected with P. berghei ANKA 6653 strain. To form a malaria infection model, the peritoneal cavity of each mouse in the study group was injected with a suspension containing 1.0x10⁵ parasite/ml. Parasitemia was determined at the seventh day of postinfection in Giemsa-stained blood smears from tail blood. The preparations were evaluated microscopically for parasitemia. Malaria infection was confirmed clinically as well as microscopically.

Isolation of mononuclear cells from mouse spleen

Mice were killed by cervical dislocation following ether anesthesia at the seventh day of infection. Their spleens were removed and mononuclear cells were isolated. After the spleen was mashed, the suspension was centrifuged and the supernatant was discarded. Erythrocytes were removed by using Tris/ammonium sulfate solution. After centrifugation, buffy coats were collected and washed in Phosphate Buffered Saline (PBS) three times and resuspended at a concentration of 2x10⁶ cells/ml in complete RPMI 1640 medium containing 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, 0.05 mM 2 mercaptoethanol and supplemented with 10% fetal calf serum (Gibco, Germany). Cell viability was 95% by the trypan blue dye exclusion assay. The same procedure was performed to the control group without Plasmodium infection (n=15).

Flow cytometric analysis of mononuclear cells

The cells were washed in PBS and stained with antibodies for 30 minutes at 4°C. Mononuclear cells (10⁶ cells/ml) were incubated with monoclonal antibodies specific for mouse CD antigens. Antibodies used were anti-mouse CD3, anti-mouse CD4, anti-mouse CD8, anti-mouse CD19, anti-mouse CD152, anti-mouse pan NK, anti-mouse CD80 (e-bioscience, USA). Flow cytometric analysis were done by using a Coulter FC500 flow cytometer (Coulter, USA).

Levels of cytokines

For cytokine assay, cardiac blood sample was collected. After centrifugation, the plasma was separated and stored at -80°C until analysis. Levels of cytokines were determined by specific enzyme-linked immunosorbent assay (ELISA) techniques according to the manufacturer’s instructions.
The concentration of cytokines was determined spectrophotometrically. The absorbance was read at 450 nm. We constructed a standard curve using cytokine standards. The cytokine concentrations for unknown samples were calculated according to the standard curve.

Statistical analysis
In control group and infected mice group, expression of cell surface markers on mononuclear cells and cytokine levels was analysed using SPSS (13.0 version) with the Mann Whitney U test. p<0.05 was considered to be significant.

Ethics
All experiments were performed in compliance with local animal ethics committee requirements (No: 2006/05.082).

RESULTS

Expression of cell surface markers on mononuclear cells
This study found a significant difference between the CD4⁺ lymphocyte, CD8⁺ T lymphocyte and NK percentages of the mononuclear cells in mice infected with *P. berghei* and those of the control groups without malaria infection. CD4⁺ lymphocyte, CD8⁺ T lymphocyte and NK percentages were statistically significantly lower in the infected mice than in the healthy control mice (p<0.05) (Fig. 1). In our study, CD19 expression found on B lymphocyte surface decreased significantly in mononuclear cells of the mice infected with *P. berghei* compared to those of the control group (p<0.05).

In addition, expression of CD152 (CTLA-4) surface antigen, which increases on the surface of activated T lymphocytes, was found to have decreased in the specimens with *P. berghei* infection compared to those of the control group. Similarly, expression of CD80 surface antigen, which increases on the surface of activated T and B lymphocytes, decreased in the infected mice compared to those of the control group which was not infected (p>0.05). Our study determined a decrease in the expression of T,B and NK cell surface markers and inhibition of these cells during infection in *P. berghei* infected mice.

Cytokine levels
This study observed that the concentrations of TNF-α, IFN-γ, IL-4, IL-10 and IL-12 in the cardiac blood sample of the infected mice were sig-

| Cytokine levels (mean ± SEM) |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | TNF-α (pg/ml)   | IFN-γ (pg/ml)   | TGF-β (pg/ml)   | IL-4 (pg/ml)    | IL-10 (pg/ml)   | IL-12 (pg/ml)   |
| Infected group (n=11) | 11.9±1.97       | 212.5±27.78     | 1601.5±188.95   | 33.7±8.99       | 112.9±18.87     | 2024.3±183.74   |
| Control group (n=15)  | 0.7±0.07        | 17.1±2.98       | 1356.6±260.88   | 0.1±0.02        | 21.7±0.72       | 849.3±92.48     |
| P values             | <0.001          | <0.001          | >0.05           | <0.001          | <0.001          | <0.001          |
nificantly more than those in uninfected controls \((p<0.001)\) (Table 1, Fig. 2). The levels of TGF-1β increased in mice infected with \(P. berghei\) compared to those of the controls, but the increase was not statistically significant \((p>0.05)\). Therefore, significant alterations in cytokine profile were determined during \(P. berghei\) infection.

**DISCUSSION**

Acute malarial infection is known to damage the normal profile of immune cells in the peripheral blood. In addition, there is much evidence showing the potential of malaria infection to cause a depletion of lymphocyte populations in the peripheral blood (Kassa et al., 2006).

In our study, there were significant decreases in the percentages of T, B and NK cells in \(P. berghei\)-infected mice group. Additionally, the expressions of CD152 and CD80 surface antigens, like the lymphocyte activation markers, were found to be markedly reduced in the \(P. berghei\)-infected group. From these data, it was thought that this suppression in immune cells might lead to a progression of infection and consequently to the death of the infected mice. This can be an important factor. Kassa et al. (2006) also reported that absolute counts of CD4+, CD8+, B and CD3+ cells and total lymphocytes were decreased very significantly during both \(P. falciparum\) and \(P. vivax\) infections. However, the NK cell count was an exception and was not affected either by \(P. falciparum\) or by \(P. vivax\) malaria (Kassa et al., 2006). As in our study, other investigators have also reported lower absolute counts of CD4, CD8, CD3, B and NK cells and total lymphocytes (Lisse et al., 1994, Hvid et al. 1997, Lee et al. 2001) during acute \(P. falciparum\) malaria. This could also be due to differences in the baseline values of the absolute counts of the immune cells of the study subjects or due to the impact of different geographical locations (Tsagaye et al., 1999, Kassu et al., 2001)

Worku et al. (1997) detected significantly lower absolute counts of NK cells in \(P. vivax\) patients than in \(P. falciparum\) patients which might suggest that this change was associated with \(P. vivax\) infection and might indicate that NK cells play a different role in \(P. falciparum\) and \(P. vivax\) infections. Down-regulation of NK cells by parasite factors could potentially be a strategy of the parasite to avoid host immune protection.

In our study, as reported in the previous studies, it was observed that during \(Plasmodium\) infection there is a significant suppression in immune cells, and this condition could be an important factor for preparing a basis for a progression of an infection. On the other hand, the determination of significant reductions in all lymphocyte populations in the infected group, obtaining samples from the infected mice in the late period and the severity of clinical outcome reveal that there...
could be a severe immune suppression in the late period of *Plasmodium* infections.

In human malaria it was shown that an appropriate ratio of proinflammatory and anti-inflammatory cytokines is important to achieve protection but also to prevent pathology (Chaiyaroj et al., 2004). Suppression of the anti-inflammatory immune response often leads to a decreased parasitemia but enhances pathology, whereas neutralization of pro-inflammatory cytokines has the potential to prevent immune pathology e.g. cerebral malaria (Singh et al., 2002). Cytokine production, TNF-α and IFN-γ, appears necessary for the inhibition of parasitemia (Schofield and Grau, 2005). Paradoxically, these cytokines are also implicated in the pathology of complicated malaria. T-cell-deficient nude mice do not develop cerebral manifestations upon parasite challenge (Finley et al., 1982). In experimental models, TNF-α has a clear causal role in the pathogenesis of both murine cerebral malaria and other features of severe disease (Marsh and Snow 1997, Rénia et al., 2007).

In our study, some proinflammatory cytokines (TNF-α, IFN-γ and IL-12) and antiinflammatory cytokines (TGF-1β, IL-4 and IL-10) were found to be high in *P. berghei*-infected mice group. The increase in TGF-1β was not statistically significant. TGF-1β has both proinflammatory and anti-inflammatory properties, depending on its environment and concentration (Omer and Riley, 1998). In the light of the present data obtained from this study, we think that both proinflammatory and antiinflammatory cytokines might be implicated in the clinical picture of *Plasmodium*-related infections.

In humans, the role of proinflammatory cytokines in malaria is less well defined. A role for the host response in malarial pathology is indicated by a correlation of increased levels of some proinflammatory cytokines and the severity of disease in humans (Lyke et al., 2004, Ong’echa et al., 2008), and the amelioration of cerebral and other pathology in rodent models by removal of TNF-α or IFN-γ (Yanez et al., 1996, Nie et al., 2007). TNF-α appears to be pivotal both in the early response to malaria and in late pathological manifestations (Kwiatkowski et al., 1990) and has been linked to disease severity and complications (Shaffer et al., 1991, Day et al., 1999). IL-12 plays an important role in the adaptive immune response to malaria (Malaguarnera and Musumeci, 2002, Lyke et al., 2004). Elevated levels of anti-inflammatory IL-10 have been reported in severe malaria (Sarthou et al., 1997). As also determined in our study, elevated serum concentrations of TNF-α have been reported during malaria, and high TNF-α concentrations correlate strongly with increasing severity of disease (Othoro et al., 1999, Luty et al., 2000, Lyke et al., 2004). Comparison of cell-mediated activity or circulating cytokine levels in children and adults in different areas of malaria transmission shows that Th1-like responses seen in childhood tend to be partially replaced by Th2-like responses in adulthood (Elghazali et al., 1995, Luty et al., 1999). The present study in the infected animals disclosed a significant increase in both Th1 and Th2 type cytokine responses. Nevertheless, we think that the samples obtained at the late period of infection may explain the increases in both cytokine responses. For this reason, our future study will focus on the investigation of cytokine levels in samples obtained at different times.

As a conclusion, important changes in immune response were observed during *Plasmodium* infection. The changes in the percentages and activation markers of T, B and NK cells, the increase in cytokine secretion and immunological changes seen particularly in the late period of infection might have significant effects on disease mortality. The full understanding of the elements and mechanisms of humoral and cell-mediated responses taking place after Plasmodium infection is very important for the pathogenesis and may provide new strategies for the prevention and treatment of malaria infection and also for vaccination in patients.

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