รายงานการวิจัยฉบับสมบูรณ์

โครงการ “การศึกษาความสัมพันธ์ระหว่างการผันแปรของระดับ cytokine และยีนของ cytokine กับพยาธิสภาพความรุนแรงของโรคมาลาเรีย”

โดย

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คณะแพทยศาสตร์ มหาวิทยาลัยศรีนครินทรวิโรฒ

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ABSTRACT

The role of cytokines in general, and the balance of pro- and anti-inflammatory cytokines in particular, in severe falciparum malaria in adults are not well defined. Functional *IL10*-1807 C/T and *TNF*-308 polymorphisms and the relative amounts of IL-10 and TNF were investigated in relation to severity of malaria in 108 and 165 Thai patients with complicated and uncomplicated malaria, respectively. The plasma IL-10 and TNF levels were determined by ELISA and the *IL10*-1802 and *TNF*-308 polymorphisms were genotyped by PCR-RFLP. The IL10 levels were significantly elevated in patients with complicated malaria in the initial stage of the disease before treatment compared to the levels in those with uncomplicated malaria (193.7 pg/ml versus 83.5 pg/ml $p < 0.0001$), while no significant difference in the TNF levels was noted between the two patient groups (80.6 pg/ml versus 80.0 pg/ml). Likewise, the levels of IL-10 to TNF ratio in patients with complicated were significantly higher than uncomplicated malaria (2.5 versus 1.12 $p < 0.0001$). The percent frequencies of IL10-1082 AA/AG/GG genotypes were 85.96/15.09/0.94 and 90.32/8.39/1.29 in patients with complicated and uncomplicated malaria, respectively. While the percent frequencies of TNF–308 GG/GA genotypes were 93.52/6.48 in complicated and 93.25/6.75 in uncomplicated malaria patients. However, no differences in *IL10*-1082 and *TNF*-308 genotype and allele frequencies between the two patient groups were seen. The exploration of individual cytokine levels and their corresponding gene polymorphisms in relation to clinical disease and previous malaria episodes would be relevant. The results on the role of pro- and anti-inflammatory cytokines and their related gene polymorphisms are essential for better understanding on the alteration of disease severity, which might facilitate the rationale design of vaccines and novel therapeutics.

Keywords : *Plasmodium falciparum*; malaria; cytokine polymorphism; IL10; TNF, inflammatory cytokine.

Abbreviations : IL-10, Interleukin 10; TNF : tumor necrosis factor (formerly tumor necrosis factor alpha), IFN-γ : Interferon gamma

บทคัดย่อ

การศึกษาความสัมพันธ์ของการผันแปรของ IL10 ยีน ตำแหน่ง –1082 A > G และ TNF ยีน ตำแหน่ง –308 G > A กับระดับของ IL-10 และ TNF ในผู้ป่วยมาลาเรียที่มีพยาธิสภาพรุนแรงจำนวน 108 ราย และไม่รุนแรงจำนวน 165 ราย ด้วยวิธี PCR-RFLP และ ELISA พบว่า IL10 –1082 และ TNF-308 genotype และ allele frequencies ในผู้ป่วยมาลาเรียที่มีอาการรุนแรงไม่มีความแตกต่างกันกับผู้ป่วยมาลาเรียที่มีอาการไม่รุนแรง อย่างไรก็ตาม ระดับของ IL-10 ในเลือดของกลุ่มผู้ป่วยมาลาเรียที่มีอาการรุนแรงสูงกว่าระดับของ IL-10 ในกลุ่มผู้ป่วยมาลาเรียที่มีอาการไม่รุนแรงอย่างมีนัยสำคัญ ในขณะที่ระดับของ TNF ในเลือดของกลุ่มผู้ป่วยมาลาเรียที่มีอาการรุนแรงและกลุ่มผู้ป่วยมาลาเรียที่มีอาการไม่รุนแรงไม่มีความแตกต่างกันอย่างมีนัยสำคัญ เมื่อศึกษาอัตราส่วนของระดับ IL-10 ต่อ ระดับ TNF (IL-10/TNF) พบว่าอัตราส่วน IL-10/TNF ในกลุ่มผู้ป่วยมาลาเรียที่มีอาการรุนแรงสูงกว่ากลุ่มผู้ป่วยมาลาเรียที่มีอาการไม่รุนแรงอย่างมีนัยสำคัญ

EXECUTIVE SUMMARY

The role of cytokines in general, and the balance of pro- and anti-inflammatory cytokines in particular, in severe falciparum malaria in adults are not well defined. Individuals who have been living in malaria endemic areas experience persistent subclinical infection, but only some develops severe disease. Growing evidence indicates that inter-individual variation in cytokine production may be reflected by polymorphism in the regulatory region of the corresponding genes. The single nucleotide polymorphism (SNP) and/or the combination of the SNPs may, therefore, influence cytokine production and in turn effect of the clinical outcomes. Therefore, the investigation on the role of IL-10, the relationship and the regulation between pro- (TNF) and anti-inflammatory cytokines (IL-10) and their gene polymorphisms will be useful for better understanding the alteration of disease severity. The cytokine allele genotypes and frequencies were determined by PCR-RFLP and the cytokines were measured by ELISA. The percent frequencies of *IL10*-1082 AA/AG/GG genotypes were 85.96/15.09/0.94 in complicated and 90.32/8.39/1.29 in uncomplicated malaria patients. While the percent frequencies of *TNF*–308 GG/GA genotypes were 93.52/6.48 and 93.25/6.75 in patients with complicated and uncomplicated malaria, respectively. The significant higher levels of IL-10 and the ratio of IL-10/TNF were seen in patients with complicated as compared to those with uncomplicated malaria. No significant difference in the levels of TNF was noted between the two patient groups.

In view of the present observation on the promoter polymorphism in relation with TNF and IL-10 levels and clinical outcome, although there were no association of the *TNF*-308G/A and *IL10*–1082G/A polymorphism with malaria severity and the related cytokines levels, the results demonstrated a significant higher levels of IL-10 and IL-10/TNF ratio in patients with complicated as compared to those with uncomplicated malaria. This indicated that the balance between Th1 and Th2 immune response or between pro-inflammatory and anti-inflammatory cytokines is important in determining the outcome of the disease. These investigations would be useful in identifying malaria severity, and may facilitate the rationale design of vaccines and novel therapeutics. The exploration of individual corresponding cytokine levels and their gene polymorphisms in relation to clinical disease and previous episodes would be relevant.

INTRODUCTION

Malaria remains a major cause of morbidity and mortality throughout much of the tropical world, with an incidence of 300-500 million clinical cases each year, and causes 1.5-2.7 million deaths (WHO, 1997). In non-immune adults, severe malaria often presents as a multisystem disorder, with features including renal failure, jaundice, acidosis, pulmonary edema, shock and coma (Miller LH, 2002). In Thailand, severe falciparum malaria is characterized by high-density parasitemia and cerebral malaria, with severe anemia occurring less frequently (Vannaphan S, 2005).

The kinetics of primary malaria infections in mice suggest that pro-inflammatory such as interferon gamma (IFN-γ) and tumor necrosis factor (TNF) are induced by innate responses mediated by monocyte/macrophage, dendritic cells (DC), natural killer cells (NK), NKT cells and/or γδ Ţ cells which limit the initial phase of parasite replication (Fell AH, 1998). So that interleukin 10 (IL10) and IL4 responses mediated by αβŢcells and B cells are required for parasite elimination in the late phase of the infection (Langhorne J, 1998).

In humans, the serum IFN-γ levels have been correlated with resistance to reinfection with *P. falciparum* (Deloron P, 1991), but the plasma IFN-γ concentrations are higher in individuals with symptomatic than in individuals with asymptomatic infections (Mshana RN, 1991). In fact, IFN-γ is the key inducer of the immune effector mechanisms that are essential for initial control of both pre-erythrocytic and blood-stage malaria infections (Good MF, 1999; Plebanski M, 2000), but there is evidence that IFN-γ levels are needed to be carefully balanced to avoid immune pathology (Artavanis-Tsakonas K, 2003). *In vivo*, TNF production is associated with parasite clearance and resolution of fever (Kremsner P, 1995), but the elevated levels of TNF (Kwiatkowski D, 1990) and IL-6 (Lyke KE, 2004) have also been associated with severe malaria. Evidence also suggests that both TGF−β and IL-10 can be produced very rapidly from innate sources during murine malaria infections and are required to down-regulate potentially pathogenic inflammatory responses once parasitemia is brought under control (Li C, 2003). The significantly higher circulating IL-10 to TNF ratios were shown in children with mild and high-density parasitemia when compared to children with malaria anemia ,while high ratios of circulating IL-4

to IFN-γ are associated with uncomplicated malaria (Othoro C, 1999; Tangteerawata P, 2007). Thus, IL-10 and IL-4 appears to play an important role in counteracting the potentially harmful host pro-inflammatory response to malaria antigens.

Tumor necrosis factor is a cytokine that initiates the inflammatory cascade, and induces the production of numerous additional mediators, with are associated with malaria sequestration (Grau GD, 1989). IL10 is an important component of the anti-inflammatory cytokine network, suppression and synthesis of pro-inflammatory cytokine (Lalani I, 1997). Polymorphisms involving the promoter region of the *TNF* and *IL10* genes, affecting transcriptional activity, have been shown to influence TNF and IL-10 production (May J, 2000; Ouma.C, 2008).

Individuals living in endemic areas experience persistent subclinical malaria infection, but only some develops severe disease. There is growing evidence that inter-individual variation in cytokine production may be reflected by polymorphism in the regulatory region of the corresponding genes (Kwaitkowski DP, 2005; Verra F, 2009). The single nucleotide polymorphism (SNP) and/or the combination of the SNPs, may therefore, influence cytokines production and in turn effect of the clinical outcomes. Most authors reported the relationship between SNPs and severity of malaria or the relationship between cytokine levels and severity of malaria. There are limited information regarding the relationship among individuals SNPs, cytokine levels and malaria severity. Furthermore, genetic variation is found among African, American, European, and Asian (Padyukov L, 2001)

Therefore, we investigate the role of IL-10, the relationship and the regulation between pro- (TNF) and anti-inflammatory cytokines (IL-10) and their gene polymorphisms in patients with complicated and uncomplicated malaria in Thailand.

MATERIALS AND METHODS

Patients with falciparum malaria

The falciparum malaria patients admitted to the Hospital for Tropical Diseases, Faculty of Tropical Medicine, Mahidol University, Bangkok were enrolled in the study with informed consent. One hundred and eight patients with complicated malaria and 165 patients with uncomplicated malaria including cerebral malaria, hyperparasitaemia, anemia, *etc* based on the WHO criteria were studied. The study

was under approval of the Ethical Committee of the Faculty of Tropical Medicine, Mahidol University.

Blood collection

The venous blood was collected in EDTA sterile tubes before treatment on admission. The plasma and packed cells were separated and stored at -20^{o}C until use. The cytokines in plasma and the allele genotypes and frequencies in cells were detected.

Cytokine allelic polymorphisms

DNA was extracted from buffy coat by phenol-chloroform extraction and analysed for single nucleotide polymorphism (SNPs) of A/G at –1082 in IL10 gene and G/A at –308 in TNF gene by polymerase chain reaction (PCR) followed by specific enzyme digestion. The PCR products were visualised on Ethidium Bromide (EtBr) stained 2% agarose gel. Amphification of both loci were performed in a total volume of 20 μl containing 2 μl genomic DNA, 2x PCR Master Mixed and 0.25 μM of each primer. The PCR amplicons were incubated for 90 min at 37°C with restriction endonucleases EcoN1 for *IL10*–1082 and with Nco1 for *TNF*–308 in buffer provided by the manufacturer (Padyukov l, 2001).

Detection of cytokines

A two-site ELISA was used to determine cytokine levels in plasma. The levels of IL-10 and TNF were estimated using optimal concentrations of monoclonal antibodies and cytokine standards according to the manufacturer's instructions.

Statistical analysis

Comparison of proportions will be made by using Chi-square analysis. The Mann-Whitney U test was applied for comparison between complicated and uncomplicated malaria groups. A *p*–value of less than 0.05 was considered to be significant.

RESULTS

IL10-1082 A/G and TNF –308 G/A genotype frequency

The frequencies of the *IL10*-1082 A/G and *TNF*–308 G/A polymorphism were determined in 108 and 165 patients with complicated and uncomplicated malaria, respectively. The allele and genotype frequencies in both groups was followed Hardy-Weinberg equilibrium. The frequencies of *IL10*-1082 AA/AG/GG genotypes were 85.96/15.09/0.94 percent in patients with complicated and 90.32/8.39/1.29 percent with uncomplicated malaria, while the percent frequencies of *TNF* –308 GG/GA genotypes were 93.52/6.48 and 93.25/6.75 in patients with complicated and uncomplicated malaria, respectively. Both *IL10*-1082 and *TNF*-308 genotype frequencies did not differ between the two patient groups.

Plasma IL-10 and TNF levels

Plasma IL-10 and TNF levels were evaluated in 108 patients with complicated and 165 uncomplicated malaria. The significant higher levels of IL-10 and IL-10/TNF were seen in patients with complicated as compared to those with uncomplicated malaria. No significant difference in the levels of TNF was noted between the two patient groups. (Table 1)

Table 1. Cytokine levels in patients with complicated and uncomplicated malaria

Characteristics	Complicated	Uncomplicated	P-*value*
IL-10 D0	193.7 ± 25.53	83.5 ± 10.74	< 0.0001
TNF D0	80.6 ± 5.57	80.0 ± 4.68	NS
IL-10/TNF D0	2.5 ± 0.29	1.12 ± 0.12	< 0.0001

Association of IL10-1082 G/A and TNF-308 G/A and plasma IL10 and TNF levels

There were no association of *IL10*-1082 G/A with plasma IL-10 levels and with IL-10/TNF ratio in both complicated and uncomplicated malaria groups. Similarly, no association of *TNF*-308G/A with plasma TNF levels and with IL-10/TNF ratio in both patient groups were found.

DISCUSSION

This study did not detect any association of the *TNF*-308 G/A and *IL10*–1082 G/A polymorphism with severity of malaria. When the association between *TNF*-308 G/A polymorphism and the circulating TNF levels as well as between *IL10*-1087 and the circulating IL-10 levels were evaluated, no association of *TNF*-308 G/A with TNF level and of *IL10*-1082 G/A with IL10 level were found. However, we observed a significant higher levels of IL-10 and IL-10/TNF ratio in patients with complicated as compared to those with uncomplicated malaria, but no significant difference in the levels of TNF was observed between the two patient groups.

Malaria-infected individuals produce large amounts of pro-inflammatory cytokines (type 1) such as TNF, IL-6, and IFNγ. This innate cytokine response is responsible for the high levels of fever and antiparasitic actions that occur within a few days of the onset of blood stage infection in nonimmune individuals (Grau GE, 1989, Kwiatkowski D, 1990). IL-10 is an anti-inflammatory (type 2) cytokine produced primarily by monocytes and T lymphocytes and is important for down-regulating expression of the pro-inflammatory immune response (Lalani l, 1997). The plasma level of TNF seems to be related to the occurrence of complications form of the disease. In children, TNF plasma levels are higher in cases of fatal malaria compared with nonfatal malaria and in complicated malaria compared with noncomplicated malaria (Grau GE, 1989, Kwiatkowski D, 1990). In contrast to such study, the median TNF plasma levels in patients with complicated and uncomplicated malaria did not differ. In the previous studies, it has been described that elevated serum and plasma TNF levels may not develop severity or clinical illness (Grau GE, 1989, Kwiatkowski D, 1990). One explanation for this may be the fact that the half-life of TNF is very short and sustained elevations may be necessary for toxic effect to develop deleterious disease condition (Kaufmann GP, 1997). The present study demonstrated the significant higher IL10 plasma levels in patients with complicated compared to uncomplicated malaria. Similar to those previously reports in various experiments, the IL-10 levels were increased in malaria patients (Ho M, 1995; Wenisch C, 1995). Earlier studies reported that high level of IL-10 associated with less effective clearance of parasite. This perhaps due to the mechanism in which IL-10 hinders the host's ability to clear parasites by suppressing pro-inflammatory cytokines possibly via antigen presentation and/or enhancing activation of regulatory

T or B cells (Urban B, 1999). On the other hand, it has been demonstrated in Ghanaian children with *P. falciparum* malaria that, low circulating IL-10 levels were found to be associated with severe malarial anaemia (Kurtzhals JA, 1998). Down regulated IL-10, the anti-inflamatory cytokines may facilitate effective clearance of parasite by pro-inflammatory cytokines, TNF. However, over-expression of TNF can promote enhanced malaria pathogenesis. Owing to downregulation of TNF by IL-10, researchers have tried to determine whether the IL-10 to TNF ratio could be a better marker of the risk of severe malaria and especially of CM, than IL-10 or TNF considered individually. In the current study, the plasma ratio of IL-10 to TNF were significantly higher in patients with complicated compared to uncomplicated malaria. In contrast with the previous studies in children, the IL-10 to TNF ratio was reduced in both Ghanaian and Kenyan children with severe malaria anaemia (Kurtzhals JA, 1998; Othoro C, 1999) and in Gabonese children with severe malaria in general, when compared to children with uncomplicated malaria fever. The balance between pro- and anti-inflammatory responses against the parasite is considered critical for clinical protection. The overproduction of both pro-inflammatory and anti-inflammatory cytokines can be responsible for disease severity and mortality.

To date, several SNPs have been identified in the promoter region of the TNF and IL10 gene (Goldfel AE, 1990; D'Alfonso S and Richiardi PM, 1994; Herrmann SM, 1998; Uglialoro AM, 1998; Hamann A, 1995; Higuchi T, 1998; Giordani l, 2003. Of these, the *TNF*-308G/A and *IL10*-1082G/A polymorphism have been reported to influence there promoter activity and to be associated with enhanced the protein production (Wilson AG, 1997; Turner DM, 1997). The present study demonstrated no differ in the major allele *TNF*-308A and *IL10*-1082A in patients with complicated and those with uncomplicated malaria. No significant association of the *TNF*-308G/A polymorphism with plasma TNF levels, and with severity of malaria. Similarly, there were no significant association of the *IL10*-1082G/A polymorphism with plasma IL-10 levels and with severity of malaria. Our results were consistent with the previous reports which demonstrated the lack of association between *IL10*-1082 gene polymorphisms and severe malaria as well as lack of association between *TNF*-308 gene polymorphisms and severe malaria in Thailand (Ohashi.j, 2002). In contrast to the previous reports in Gabonise children infected with malaria, Ganoise children with homozygous for *TNF* wile type promoter polymorphism had higher IL-10/TNF ratios compared to those with other TNF promoter variants (May J, 2000). While the study

in Kenyan children showed that haplotypes of *IL10* varients affect the plasma level of IL-10 and IL-10 over TNF in malaria patients (Ouma C, 2008). The reason for genetic disparities and varied degree of association with disease susceptibly can be attributed to highly polymorphic chromosomal location, biological effect and extensive linkage disequilibrium to the HLA locus, such differences are likely to exist. In addition, the different ages, ethnicity and geographical differences in the studied population can also contribute to the genetic association and disease outcome.

In view of present observation of promoter polymorphism in relation with TNF and IL-10 levels and clinical outcome, although there were no association of the *TNF*-308G/A and *IL10*–1082G/A polymorphism with the severity of malaria and the related cytokines levels, the results demonstrated a significant higher levels of IL10 and IL-10/TNF ratio in patients with complicated as compared to those with uncomplicated malaria. This indicated that the balance between Th1 and Th2 immune response and between pro-inflammatory and anti-inflammatory cytokines is important in determining the level of malaria parasitemia, the disease outcome and the rates of recovery. These investigations would be useful in identifying malaria severity, and may facilitate the rationale design of vaccines and novel therapeutics.

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SIGNIFICANCE OF THE RESEARCH

1. The present study will be published in the international journal.

2. The exploration in cytokine responses in malaria patients may provide clue for better understanding of the immune responses. The results may valuable in prescribing appropriate anti-malarial drugs or deciding which patients are most likely to benefit from a vaccine or life threatening.

ภาคผนวก

The manuscript is now being prepared for publication in international journal and as the report above.