International Journal of Genetics and Molecular Biology Vol. 2(5), pp. 67-71, May 2010
Available online at http://www.academicjournals.org/IJGMB
ISSN 2006-9863© Academic Journals

Full Length Research Paper

Lack of association between - 308 tumor necrosis factor polymorphism and susceptibility to cerebral malaria among Central Sudanese children

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Accepted 13 April 2010

Up to 500 millions are affected by malaria parasite each year and only 1% of them develop severe clinical forms of the disease. No full satisfactory explanation for the fact that only small proportion of malaria infected individuals develops severe clinical phenotypes while others don't. The aims of the present study is to assess the role of TNF-α - 308 G>A in predisposing to cerebral malaria in children in Central Sudan. 109 children admitted with cerebral malaria (CM) were enrolled in this study (Mean age 6.1± 3.3 years old). The onset of cerebral malaria in the study subjects started in the first two years of life with no gender effect and the highest incidence of the disease was at the age group (4 - 6 years old). The allele frequencies in control group (n= 109) were 0.91 for TNFα -308G (TNF1) and 0.09 for TNFα -308A (TNF2) and the allele frequencies in 93 CM subjects were 0.95 for TNFα -308G (TNF1) and 0.05 for TNFα -308A (TNF2). The distribution of TNFα -308 genotypes in normal group was consistent with the Hardy-Weinberg equilibrium. No TNF2 homozygote was observed among CM subjects. However, the distribution of TNFα -308 genotypes and alleles did not differ significantly between CM patients and controls (P = 0.271). These data suggest that TNF2 is not associated with predisposition to CM in Central Sudanese children. Further studies for confirmation of this finding in other regions of Sudan are required.

Key words: Cerebral malaria, genetic susceptibility, cytokine, TNF.

INTRODUCTION

Host genetic back-ground is the key in the control of the internal environment which the parasite faces during malarial infection. The major disadvantage of being parasitized should favor the survival of those host alleles that confer some protection against the parasite. During *P. falciparum* infection, malaria toxins are secreted or released from parasites at the late stages (trophozoite and especially the schizont stage) (Schofield et al., 1993; Martinez et al., 2000; de Souza et al., 2002). These products either directly damage host tissues or, more importantly stimulate the production of host cytokines (Jakobsen et al., 1995; Tachado et al., 1999). Moderate amounts of cytokines such as tumor necrosis factor alpha (TNF-α), gamma interferon (IFN-γ), and interleukin-1 (IL-1) are necessary for fighting invading microorganisms. However, overproduction of these cytokines can also be very harmful and can cause severe disease (Clark et al., 1989; Clark et al., 1991).

TNF-α causes fever and disturbance of the immune

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Figure 1. The locations of the three cities, (Wad Medani, Sinnar and Singa).

system. TNF-α and IFN-γ upregulate endothelial receptors such as ICAM-1 and probably redistribute other receptors, such as CD31 on endothelium, which may enhance parasite sequestration (Dodoo et al., 2002).

Tumor necrosis factor alpha (Engwerda et al.) is a potent cytokine with a wide range of proinflammatory activities (Grau et al., 1989). Classically, it is produced by monocytes and macrophages, although other cell types, such as T and B cells produce significant amounts and mediates its functions by binding TNF receptors, TNFR1 and TNFR2 (Carballo and Blackshear, 2001). Over-production of TNF-α is considered a major pathogenic mechanism responsible for fever and tissue lesions in P. falciparum malaria. Clinical studies have demonstrated an association between TNF-α levels and malaria severity (Grau et al., 1989; Kwiatkowski 1990; Awasthi et al., 2008; Sinha et al., 2008). Study in Gambian children had shown that TNF-α level was higher in patients with CM than those having mild malaria. A similar finding had been observed in Kenyan children (Knight et al., 1999). Further, TNF-α level were highest in children who died or developed severe neurological sequelae (Kwiatkowski, 1990; Tchinda et al., 2007).

Also carrying TNF2 increases the risk of reinfection with the malaria parasite P. falciparum by decreasing the intervals between infections (Meyer et al., 2002). Several polymorphisms had been described in the promoter region of TNF-α that effect its transcription (Knight et al., 1999). The one of interest is a group of SNPs located at -238, - 308 and - 376 with respect to transcriptional start site. All are transitions of guanine for adenine. TNF-308 polymorphism generates the TNF1 and TNF2 alleles, respectively. The TNF2 allele is associated to a high in vitro TNF-α expression level (Wilson et al., 1997). This has also been linked to an increased susceptibility and severity, for a variety of illnesses including CM (Abraham and Kroeger, 1999).

The same allele has been associated with susceptibility to various other infectious diseases including leishmaniasis, lepromatous leprosy, trachoma and meningococcal septicemia (Knight and Kwiatkowski 1999). TNF -376 polymorphism is located in a region of multiple protein interactions. The less common allele, TNF-376A, acts to influences the structural conformation of the region with adoption of higher degree of curvature. TNF-376A allele is in negative disequilibrium with TNF-308A and in strong linkage disequilibrium with TNF-238A, such that individual with TNF376A form a subset of individuals possessing TNF238A. TNF-376A allele is associated with susceptibility to cerebral malaria in both Gambian and Kenyan children.

This association is entirely independent of that observed for the TNF-308A allele (McGuire, Knight et al. 1999). The functional significance of the rare TNF-238A allele is not clear. Although, in a case-control study in The Gambia found severe malarial anemia, but not cerebral malaria, associated with the TNF-238 A allele (McGuire, Knight et al. 1999). Considerably, more evidences are accumulating to indicate the important of host factors in determining the clinical presentations of malaria infection (WHO, 1990). The full knowledge of these factors is important not only to understand how the disease evolves within an individual, but is also of great important to understand, from an epidemiological point of view, how it behaves in a given population. Most of the genetic studies in CM were done in other African countries and few in Sudan.

MATERIALS AND METHODS

Study area and subjects

The study was carried out during the period 1999 - 2003 during the rainy seasons in three hospitals located in cities of Wad Medani, Sinnar and Singa in Central Sudan. Most of severe malarial cases in this area are admitted into one of these three hospitals. 30% of all admitted patients are due to severe malaria (WHO, 2002). These cities lie along the Blue Nile; Wad Medani lies about 184 km south to Khartoum, the capital of Sudan, within the largest irrigated Gezira Agricultural Scheme, Sinnar lies immediately down the Sinnar dam which irrigates Gezira Agricultural Scheme while Singa lies about 120 km down Damazein dam and is bypassed with many canals originated from the dam (Figure 1). Thus, the whole area is a continuous breeding focus for the malaria vector, Anopheles mosquito. This area is endemic for P. falciparum with high seasonal incidence during the rainy season from August to November. A second peak of transmission follows during January to March. Thus the data was collected during the both peaks of transmission. Ethical approval was obtained both from the Federal Ministry of Health in Khartoum and the University of Gezira Research Committees. Consent was taken from the guardians or parents of all study subjects. Children were clinically examined by a

Table 1. The Laboratory findings in study subject.

Investigation	Number	Mean± SD
TWBCs (cell/dl)	100	6370 ± 2787
Hb (gm/dl)	100	8.1 ± 2.1
RBS (gm/dl)	100	91.85 ± 33.7
Blood Urea (gm/dl)	100	29.95 ± 17.75
CSF Glucose (gm/dl)	100	57.29 ± 7.7
CSF Protein (gm/dl)	100	23.23 ± 12.29
CSF cell (cell/dl)	100	7 ± 6

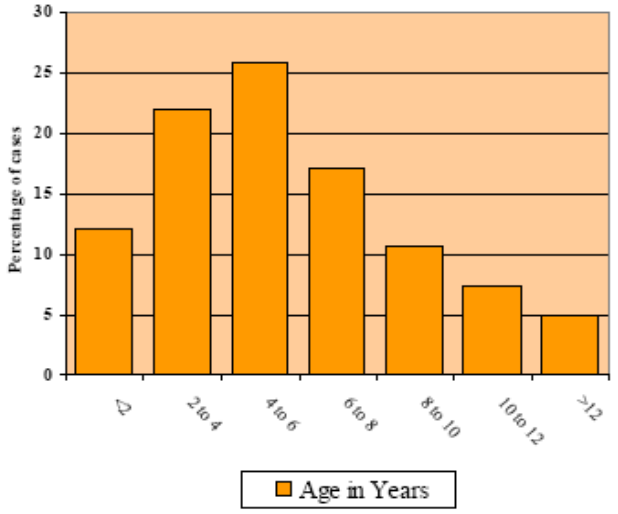

Figure 2. Distribution of study subjects according to age.

Pediatrician and CM was confirmed according to WHO criteria for diagnosis of cerebral malaria. One hundred and nine children were diagnosed as having cerebral malaria (CM). 42 (39%) from Wad Medani, 59 (54%) from Sinnar and 8 (7%) from Singa. One hundred and nine children were selected for case-control analysis from healthy pupils of different schools from the three cities or from other patients from the three hospitals who were admitted with other diseases other than CM and never had CM. They were matched in age, sex and ethic group. The mean age of the control group was 6 year.

Screening of TNF-α - 308 G>A in CM cases and controls using PCR-RFLP

Detection of TNF-α - 308 polymorphism was performed by PCR-RFLP genotyping method. This was done by amplification of a 107 bps DNA fragment flanking the polymorphic site in a total volume of 30 µl as described above using primer pair TNFAI (5`-AGGCAATAGGTTTTGAGGGCCAT-3`) and TNFA2 (5`-TCCTCCCTGCTCCCATTCCG-3`). PCR conditions as an initial denaturation 95°C for 10 min, followed by 35 cycles of 94°C as melting temperature for one minute, 46°C as annealing temperature for 45 sec and 72°C as a prolongation temperature for 45 sec, then a final prolongation step at temperature 72°C for 10 min. 2 µl PCR product was digested overnight at 37°C in a total volume of 15 µl with 2.5 U Nco I, 1.5 µl 10 x Buffer Y^{+}/Tango™ (330 mM Tris acetate (pH 7.9), 660 mM

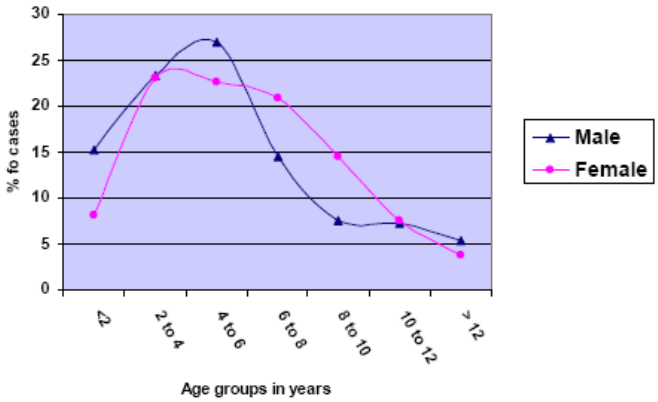

Figure 3. Distribution of study subject according to age and sex.

potassium acetate, 100mM magnesium acetate, 1 gm/ml BSA (MBI Fermentas) and deionized water. The digested DNA was loaded on a 10 % Non-denaturizing polyacrylamide gel and electrophoresed at 140 V for one hour. Then the gel was stained in 1µg/ml ethidium bromide solution for 10 -15 min and visualized with UV light under Gel Documentation System (GDS).

Statistical analysis

Statistical analyses were performed with the SPSS (version13.0) package. Data were expressed as mean ± SD, Frequencies. TNF-α -308 polymorphism genotype was calculated by counting the allelic pair composition of the gene. Allele frequencies were determined by counting the number of chromosomes bearing an allele. Association between genotypes and CM assessed using chi square and odds ratio with confidence intervals (CI 95%) which are interpreted as the relative risk of disease for CM patient compared with control. $P<0.05$ was taken as the level of significance.

RESULTS

The presenting biochemical findings of the study subjects (n =109) on admission were shown in (Table 1). The mean age of the study subjects was (6.1 ± 3.3) years old; the minimum age was 1 year old and the maximum one was 15 years old. 59 (54.5%) were males and 50 (45.5%) were females with no significant difference between them. (P = 0.77). The highest incidence of the disease was found among the age group (4 - 6 years) (Figure 2). Although, both sexes showed different peaks of incidence, for males at the 5th year and for females at the 3rd year of age with no significant differences (P = 0.054) (Figure 3). TNF-α -308 polymorphism was screened using PCR-RFLP method (Figure 4). The allele frequencies in control group (n= 109) were 0.91 for TNFα-308G (TNF1) and 0.09 for TNFα-308A (TNF2) and the allele frequencies in 93 CM subjects were 0.95 for TNFα-308G (TNF1) and 0.05 for TNFα-308A (TNF2). The distribution of TNFα -308 genotypes in normal group was consistent with the Hardy-Weinberg equilibrium. No TNF2

Table 2 The frequencies of TNFα-308 in CM patients and controls

Alleles		Genotypes			TNFα-308
TNF2	TNF1	TNF2/ TNF2	TNF1/ TNF2	TNF1/ TNF1	
0.09	0.95	0(0.0%)	9(09.7%)	84(90.3%)	Control
0.05	0.91	2(1.8%)	15(13.8%)	94(84.4%)	CM Patients
NS			NS		*P*

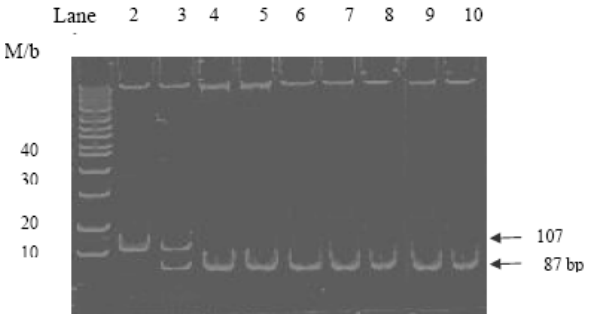

Figure 4. Screening of TNF- α-308 polymorphism using PCR-RFLP method: Lane 1: M: 100-bp DNA marker. Lane 2: homozygous for TNF2, Lane 3 is heterozygous TNF1/TNF2 while lanes 4, 5, 6, 7, 8, 9 and 10 are homozygous for TNF1.

homozygote was observed among CM subjects. However, the distribution of TNFα-308 genotypes and alleles did not differ significantly between CM patients and controls (P = 0.271) (Table 2).

DISCUSSION

The mean age of study subjects was 6.1±3.3 years with no significant gender variation, although, the number of cases in females was lower than in males below the age of six years and starts to become higher after that. The distribution of the patients according to age and sex, as shown in (Figure 2) has indicated that the onset of CM in both sex starts in the first two years of life but the number of cases increases rapidly till the age of 5 years in males and 3 years in females. Thereafter, starts to drop. The low number of CM cases in females below the age of 6 years may be explained either by; below this age females may acquire immunity earlier than males or probably they are at high risk of early dying of CM at home and fewer attend hospitals. The distribution of TNFα-308 G/A genotypes in the control group as shown in (Table 1) was consistent with the Hardy-Weinberg equilibrium. No homozygozity of TNF2 was observed in this study subjects, however, the distribution of TNFα -308 genotypes did not differ significantly between CM patients and controls (P = 0.271). Our findings indicate no association between CM and TNFα-308 G/A polymorphism. The TNF2 allele frequency was found to be higher in the healthy control (0.09) than in CM cases (0.05) however, the difference was not statistically significant. Similar results were reported; in Gabon, where there was absence of both homozygozity and association between TNF2 and malaria complications (Meyer, May et al. 2002) and in Thai, lack of association of TNF2 promoter polymorphism with disease severity (Hananantachai et al., 2001). This finding is not consistent with the results reported by several studies in which TNF2 allele confers the risk for CM (Brinkman et al., 1995; Knight and Kwiatkowski, 1999). In Gambia, children who were homozygote for the TNF2 allele had a sevenfold increased risk of CM or fatal outcome course and in Sri Lankans; TNF2 was associated with two to three time higher risk for severe malaria (McGuire et al., 1994; McGuire et al., 1999; Wattavidanage et al., 1999). The absence of homozygozity for TNF2 in CM subject in this study and the low frequency in the control group may be either due to relatively smaller sample size as according to TNF2 allele frequency (0.09) homozygozity was expected at a frequency of 0.008 or may be due to the strong negative selective pressure for TNF2 as children who are homozygote for this allele are more susceptible to several severe infectious diseases including malaria and most probably may die immediately after onset of the disease and before attending hospital and rarely reach reproductive age.

The relatively high prevalence of the non-beneficial TNF2 allele in this study population (0.09) suggests a heterozygote advantage. Possibly the TNF2 allele in homozygote status leads to increased constitutive expression of TNF α which could be harmfully to patients (Wilson et al., 1997; Abraham and Kroeger 1999). TNF-α might enhance disease either directly or through over-stimulation of the cytokine cascade (Clark et al., 1989). However, in heterozygote, the expression of TNFα may be more balanced than both homozygotes TNF1 or TNF2 and may confer protective measures against several infectious diseases other than malaria.

This explanation is supported by the observation in study in Tanzania where infants who were heterozygous for the TNF alpha -308 polymorphism appeared to have fewer febrile episodes when they were free of parasites (Stirnadel et al., 1999). This may explain the relatively high TNF2 allele frequency (0.09) remaining in our study population.

REFERENCES

Abraham LJ, Kroeger KM (1999). "Impact of the -308 TNF promoter polymorphism on the transcriptional regulation of the TNF gene: relevance to disease." J. Leukoc. Biol., 66(4): 562-566.

Awasthi GS, Singh S, Dash AP, Das A (2008). "Genetic characterization and evolutionary inference of TNF-alpha through computational analysis." Braz. J. Infect. Dis., 12(5): 374-379.

Brinkman BM, Zuijdeest D, Kaijzel EL, Breedveld FC, Verweij CL (1995). "Relevance of the tumor necrosis factor alpha (TNF alpha) -308 promoter polymorphism in TNF alpha gene regulation." J. Inflamm., 46(1): 32-41.

Carballo E, Blackshear PJ (2001). "Roles of tumor necrosis factor-alpha receptor subtypes in the pathogenesis of the tristetraprolin-deficiency syndrome." Blood, 98(8): 2389-2395.

Clark IA, Chaudhri G, Cowden WB (1989). "Roles of tumour necrosis factor in the illness and pathology of malaria." Trans. R. Soc. Trop. Med. Hyg., 83(4): 436-440.

Clark IA, Rockett KA (1991). "Role of TNF in cerebral malaria." Lancet, 337(8736): 302-303.

De Souza JB, Todd J, Krishegowda G, Gowda DC, Kwiatkowski D, Riley EM (2002). "Prevalence and boosting of antibodies to Plasmodium falciparum glycosylphosphatidylinositols and evaluation of their association with protection from mild and severe clinical malaria." Infect. Immun., 70(9): 5045-5051.

Dodoo D, Omer FM, Todd J, Akanmori BD, Koram KA, Riley EM (2002). "Absolute levels and ratios of proinflammatory and anti-inflammatory cytokine production in vitro predict clinical immunity to Plasmodium falciparum malaria." J. Infect. Dis., 185(7): 971-979.

Engwerda CR, Mynott TL, Sawhney S, De Souza JB, Bickle QD, Kaye PM (2002). "Locally up-regulated lymphotoxin alpha, not systemic tumor necrosis factor alpha, is the principle mediator of murine cerebral malaria." J. Exp. Med., 195(10): 1371-1377.

Grau GE, Lambert PH, Vassalli P, Piguet PF (1989). "Tumor necrosis factor (TNF) and pathology; its relationships with other cytokines]." Schweiz Med Wochenschr, 119(49): 1756-1761.

Grau GE, Taylor TE, Molyneux ME, Wirima JJ, Vassalli P, Hommel M, Lambert PH (1989). "Tumor necrosis factor and disease severity in children with falciparum malaria." N. Engl. J. Med., 320(24): 1586-1591.

Hananantachai H, Patarapotikul J, Looareesuwan S, Ohashi J, Naka I, Tokunaga K (2001). "Lack of association of -308A/G TNFA promoter and 196R/M TNFR2 polymorphisms with disease severity in Thai adult malaria patients." Am. J. Med. Genet., 102(4): 391-392.

Jakobsen PH, Bate CA, Taverne J, Playfair JH (1995). "Malaria: toxins, cytokines and disease." Parasite Immunol. 17(5): 223-231.

Knight JC, Kwiatkowski D (1999). "Inherited variability of tumor necrosis factor production and susceptibility to infectious disease." Proc. Assoc. Am. Physicians., 111(4): 290-298.

Knight JC, Udalova I, Hill AV, Greenwood BM, Peshu N, Marsh K, Kwiatkowski D (1999). "A polymorphism that affects OCT-1 binding to the TNF promoter region is associated with severe malaria." Nat. Genet., 22(2): 145-150.

Kwiatkowski D (1990). "Tumour necrosis factor, fever and fatality in falciparum malaria." Immunol. Lett., 25(1-3): 213-216.

Martinez AP, Margos G, Barker G, Sinden RE (2000). "The roles of the glycosylphosphatidylinositol anchor on the production and immunogenicity of recombinant ookinete surface antigen Pbs21 of Plasmodium berghei when prepared in a baculovirus expression system." Parasite Immunol., 22(10): 493-500.

McGuire W, Hill AV, Allsopp CE, Greenwood BM, Kwiatkowski D (1994). "Variation in the TNF-alpha promoter region associated with susceptibility to cerebral malaria." Nature, 371(6497): 508-510.

McGuire W, Knight JC, Hill AV, Allsopp CE, Greenwood BM, Kwiatkowski D (1999). "Severe malarial anemia and cerebral malaria are associated with different tumor necrosis factor promoter alleles." J. Infect. Dis., 179(1): 287-290.

Meyer CG, May J, Luty AJ, Lell B, Kremsner PG (2002). "TNFalpha-308A associated with shorter intervals of Plasmodium falciparum reinfections." Tissue Antigens, 59(4): 287-292.

Schofield L, Vivas L, Hackett F, Gerold P, Schwarz RT, Tachado S (1993). "Neutralizing monoclonal antibodies to glycosylphosphatidylinositol, the dominant TNF-alpha-inducing toxin of Plasmodium falciparum: prospects for the immunotherapy of severe malaria." Ann. Trop. Med. Parasitol., 87(6): 617-626.

Sinha S, Mishra SK, Sharma S, Patibandla PK, Mallick PK, Sharma SK, Mohanty S, Pati SS, Mishra SK, Ramteke BK, Bhatt R, Joshi H, Dash AP, Ahuja RC, Awasthi S; Indian Genome Variation Consortium, Venkatesh V, Habib S (2008). "Polymorphisms of TNF-enhancer and gene for FcgammaRIIa correlate with the severity of falciparum malaria in the ethnically diverse Indian population." Malar. J., 7: 13.

Stirnadel HA, Stöckle M, Felger I, Smith T, Tanner M, Beck HP (1999). "Malaria infection and morbidity in infants in relation to genetic polymorphisms in Tanzania." Trop. Med. Int. Health, 4(3): 187-193.

Tachado SD, Mazhari-Tabrizi R, Schofield L (1999). "Specificity in signal transduction among glycosylphosphatidylinositols of Plasmodium falciparum, Trypanosoma brucei, Trypanosoma cruzi and Leishmania spp." Parasite Immunol., 21(12): 609-617.

Tchinda VH, Tadem AD, Tako EA, Tene G, Fogako J, Nyonglema P, Sama G, Zhou A, Leke RG (2007). "Severe malaria in Cameroonian children: correlation between plasma levels of three soluble inducible adhesion molecules and TNF-alpha." Acta Trop., 102(1): 20-28.

Wattavidanage J, Carter R, Perera KL, Munasingha A, Bandara S, McGuinness D, Wickramasinghe AR, Alles HK, Mendis KN, Premawansa S (1999). "TNFalpha*2 marks high risk of severe disease during Plasmodium falciparum malaria and other infections in Sri Lankans." Clin. Exp. Immunol., 115(2): 350-355.

WHO (2002). "R O L L B A C K MALARIA." Regional_Office for the Eastern Mediterranean: pp. 7-8.

Wilson AG, Symons JA, McDowell TL, McDevitt HO, Duff GW (1997). "Effects of a polymorphism in the human tumor necrosis factor alpha promoter on transcriptional activation." Proc. Natl. Acad. Sci. U S A, 94(7): 3195-3199.