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Full Length Research Paper

Analysis of TNF-α and IL-10 gene polymorphisms in Zimbabwean children exposed to malaria

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Single nucleotide polymorphisms within the cytokine genes, TNF-α (-308 G/A), and IL-10 (-1082 A /G and -819 T/C) associated with protection and susceptibility to parasitic infections were examined in samples from school aged children in the Eastern district of Zimbabwe. Whole blood specimens were obtained from 491 children between the ages of 5 – 16 years, of which 26.9% were infected with *Plasmodium falciparum* and 73.1% were not infected. Genotyping was carried out using the amplification refractory mutation system-polymerase chain reaction (ARMS-PCR). The prevalence of TNF-α genotypes GG, GA and AA associated with low, intermediate and high cytokine production was 80, 19 and 2%, respectively. Wild type alleles TNF-α -308G* predominated in both infected and uninfected individuals in the study. For IL-10 (position -819) the distribution of wild-type alleles CC*, heterozygotes and mutant carriers TT* was 64, 27 and 9%, respectively, and a similar analysis of the polymorphisms on position -1082 for IL-10 revealed that most of the samples were heterozygotes (95%). There was no statistically significant difference in the frequencies of polymorphisms on TNF-α (-308G/A) (X^2 = 1.820; p = 0.403), IL-10 (-1082 A/G) (X^2 = 0.242; p = 0.623) and IL-10 (-819C/T) among children infected with *P. falciparum* and those without infection. Finally, the high prevalence of heterozygotes would suggest moderate to high IL-10 responses in the population analyzed and that an anti-inflammatory environment dominates when faced with acute *P. falciparum* infection in the samples analyzed.

Key words: Polymorphism, cytokines, *Plasmodium falciparum*, malaria.

INTRODUCTION

Parasitism is a serious health problem in Zimbabwe, and most probably in most tropical and subtropical regions. It is estimated that about 40% of a total population of 12.7 million Zimbabweans may be potentially exposed to different parasite densities each year. Of the population at risk, malaria tops the scale with about 44% exposed to negligible risk, 27% to epidemic risk and 29% to endemic risk (Ministry of Health, 2001). On average, 3 million clinical episodes of malaria and 1 500 deaths are recorded annually, in Zimbabwe (Ministry of Health, 2001).

Evidence is accumulating that the pathology observed in malaria and other parasitic cases is not caused directly by parasite products but by normal components of the immune response (Aikawa et al., 1990; Kwiatkowski et al., 2000; McGuire et al., 1994) especially cytokines like TNF-α/β, IFN-γ, TGF-β, IL-1, IL-4, IL-10, IL-12 and IL-6

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(Day et al., 1999; Kwiatkowski, 1999; Mazier et al., 2000; Upperman et al., 2005). The virulence of malaria appears to be inversely related to the capacity of the parasite to induce production of protective cytokines where TNF-α and IFN-γ are central to this process (Grau et al., 1989; Kwiatkowski, 1999). Furthermore, cytokines are thought to induce severity in malaria and other parasitic infection with production of free radicals that have an impact on the immuno-regulatory cells. Transmission patterns are core to the severity of infection and sometimes this is related to host immune development (Aikawa et al., 1990; Theander, 1992). Host response to infections differs amongst individuals and this information is essential in the development of vaccines. Immunological markers of susceptibility and resistance to infection are often useful in predicting progression and development of disease severity (Riley et al., 2006). The link of the cytokine levels to isotype production and dominance requires further elucidation. This study looked at the frequencies of major single nucleotide polymorphisms of essential cytokines that are involved in pro- and anti-inflammatory responses and relationship to plasma levels. The overall goal being to understand immune mechanism during infection and the immune response in individuals who reside in a malaria endemic area but resist development of clinical illness. Results from this study should give insight into understanding infection patterns, protective indices of immunity and would be of value to vaccine development and the communities resident in areas of high seasonal malaria.

MATERIALS AND METHODS

Study area and population

The study was conducted among 491 primary school children (aged 6 - 17 years) living in Burma Valley commercial farming area. Burma Valley borders with Mozambique and is located in Mutare district, Manicaland Province.

Study design, inclusion and exclusion criteria

A cross sectional study that involved screening of study participants for *Plasmodium falciparum* was conducted. This was part of the longitudinal study investigating the distribution of mixed infection with schistosomes, soil transmitted helminths and *P. falciparum* among primary school children living in rural and commercial farming areas in Zimbabwe. All children attending each primary school were eligible. Demographic data that includes age, gender and the village where participants lived was recorded onto a questionnaire.

Blood collection and detection of *P. falciparum*

Approximately, 5 ml of venous blood was collected into EDTA tubes for thick blood smears for malaria diagnosis and DNA extraction for cytokine single nucleotide polymorphism (SNPs) determination and serum cytokine assays. *P. falciparum* was diagnosed by microscopic examination of thick blood smears stained using the Giemsa technique (Cheesbrough, 1998). The presence of either ring forms or gametocytes was conclusive diagnosis of *P. falciparum* infection and the parasites were counted against 200 leukocytes. Children diagnosed positive for *P. falciparum* were treated with a combination of chloroquine, sulphadoxine and pyrimethamine (SP) according to local malaria case management guidelines, at that time (EDLIZ, 2006). Treatment for malaria was based on either clinical symptoms or parasitological results.

Ethical consideration

The University of Zimbabwe and The Medical Research Council of Zimbabwe (MRCZ/A/993) gave ethical approval for the study. Inclusion of children into the study took place after free individual, parental and school authority informed consent.

Cytokine assays

The serum levels of the cytokines TNF-α and IL-10 were evaluated by indirect enzyme-linked immunosorbent assays (ELISA), using pairs of cytokine-specific monoclonal antibodies provided by the commercially available test (BD, Biosciences - Pharmingen, San Diego, CA, USA). All tests were performed according to the manufacturer's instructions. Each plate included a standard curve of recombinant human cytokine run in parallel with the samples, and the limit of detection was 2000 *p*g/ml. All samples were measured in duplicate, and the mean of the two values of optical density was used for all analyses.

DNA extraction genotyping TNF-α and IL-10 genes

Blood collected into EDTA tubes was used for DNA extraction using the FlexiGene (cat #. 51204) following the manufacturer's instructions. Frozen blood was thawed at 37°C with constant agitation. The DNA concentration was determined using UV spectrophotometer and the sample was kept frozen at -20°C, only to be thawed just before performing PCR amplifications. IL- 10 -1082, -819 and TNF-α -308 were typed using amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) as described by Perrey et al. (1999). The primers used (Table 1) were obtained from Sigma Genosys, USA and Taq DNA polymerase (Invitrogen, USA).

The thin walled 0.2 ml PCR tubes strips and lid chains were obtained from Sarstedt, Germany. Briefly, two different 5′ primers respectively specific for wild type and mutant separately mixed to a 3′ generic primer, with a final concentration of polymerase, 200 µM each deoxynucleotide, 1× reaction buffer and 0.5 µM of each specific primer mix. About 5 µl of primer mix A (mixture of primer and generic primer) was added into the specific tubes for each sample, while primer mix G (mixture of generic primer and primer G) was added into the G tube. Five microlitres of about 30 –150 ng of template DNA was finally added into the specific tubes and the tubes loaded onto a thermocycler (GeneAmp® PCR System 9700). ARMS-PCR for amplification of IL10 (–1,082 G/A, –819 C/T) alleles and TNF-α (−308 G/A) alleles was used, under the following conditions: amplification consisted of a 1-min denaturation step at 95°C; 10 cycles of 15 s at 95°C and 50 s at 65°C and 40 s at 72°C; and 20 cycles of 20 s at 95°C, 50 s at 59°C, and 30 s at 72°C, followed by cooling to 4°C.

Detection of amplicons

The PCR products were detected by electrophoresis on 2% agarose gels prepared in 1x TBE (45 mM Tris-borate, 1 mM EDTA)

Table 1. List of wild type, mutant and generic primer sequences used in the determination of the single nucleotide polymorphisms.

Cytokine	*nt*	Mutation	Primers (5′-3′)	Product size (bp)
TNF-α	-308	G → A	A: AGG TTT TGA GGG GCA TTG G: AGG TTT TGA GGG GCA TGA Generic: CAG CGC AAA ACT TCC TTG GT	184
IL-10	-1082	A → G	A: TAA GGC TTC TTT GGG AG G: TAA GGC TTC TTT GGG AA Generic: TAA ATA TCC TCA AAG TTC C	258
IL-10	-819	C → T	C: CCC TTG TAC AGG TGA TGT AAC T: ACC CTT GTA CAG GTG ATG TAA T Generic : AGG ATG TGT TCC AGG CTC CT	233

Table 2. Correlation analyses of cytokine levels, parasitaemia and cytokine genotypes. Pearson's correlation test was used to examine statistical correlations.

Parameter correlation	Parasitaemia (n)	IL-10 Concentration	TNF-α concentration
Parasitaemia	n/a	0.040 (409); p= 0425	r = 0.036 (405); p= 0.475
TNF-α -308 genotypes	r = 0.039 (397); p =0.440)	n/a	0.867 (397); P <0.001
IL-10 (1082) genotypes	r = 0.0258 (407); p = 0.575	0.156 (407); p<0.001	n/a
IL-10 (819) genotypes	r =0.019 (407); p = 0.709	R=0.882 (409); p< 0.001	n/a

buffer at pH 8.3 and stained with ethidium bromide (0.5 μg/ml). The products of wild type primer and mutant primer were loaded next to each other. Electrophoresis was carried out at constant voltage of 90 - 120 V for 45 min. The reference 100 bp DNA ladder (BioLabs, New England) was included in the middle row of each 50 loads. The gels were viewed on a UV trans-illuminator (eagle eye). Images were printed and presence or absence of mutation scored from the printouts.

Statistical methods

Data was analyzed using Statistical Package for Social Scientists (SPSS) version 8.0 for Windows, SPSS inc. Chicago, USA. Analysis of variance was used to determine the difference of haemoglobin levels in different infection status. Pearson correlation analyses were performed to determine correlations among parasitaemia, cytokine levels and SNPs. Statistical significance for all analysis was determined at 5% alpha level.

RESULTS

Parasitology

Four hundred and ninety one (491) children were recruited into the study and 50.4% of these were males. The overall mean (SD) age for the study population was 10.3 years (2.3), range (6 - 17 years). Mean ages (95% CI) for males and females were 10.5 years (10.4 - 10.7 years) and 10.0 years (9.9-10.1 years), respectively. The voluntary and invasive (venous blood collection) nature of our study influenced compliance of participants; hence not all children were able to provide blood samples. The prevalence of malaria was 26.9% (132/491). Malaria was equally distributed between males and females.

Only *P. falciparum* species were found in the study subjects. The study population had various levels of parasitaemia but all fell in the mild malaria category as determined by the quantitative diagnosis (mean = 1627 parasites/μl of blood, SxSE = 810). There was however no correlation between genotypes and parasitaemia with correlation coefficient of r; (r = 0.039; p = 0.440), (r = 0.0258; p = 0.575) and (r = 0.019; p = 0.709), respectively for TNF-α (-308), IL-10 (-1082) and IL-10 (-819). Analysis of parasite density against cytokine levels revealed lack of statistically significant difference for all cytokine genotypes (TNF-α -308, p = 0.391), IL-10 -1082, p = 0.219) (Table 2). Correlation analysis of TNF-α (-308) IL-10 (-1082) and IL-10 (-819) showed no significant relationship between parasite density and genotypes at each of the three loci studied.

Effect of genotype on serum concentrations of *TNF-α* and IL-10 cytokines

Circulating concentrations of TNF-α and IL-10 were

Table 3. Allele and genotype frequencies obtained for the TNF-α and IL-10 genes in the study population.

Cytokine polymorphism	Allele/Genotype	Frequency n (%)		
		Infected	Uninfected	Total population
	G	185 (87)	522 (90)	707 (89)
	A	27 (13)	60 (10)	87 (11)
TNF-α -308G/A	GG	82 (77)	234 (80)	316 (80)
	GA	21 (20)	54 (19)	75 (19)
	AA	3 (3)	3 (1)	6 (2)
	A	118 (53)	311 (53)	492 (53)
	G	104 (47)	281 (47)	385 (47)
IL-10 1082 A/G	AA	7 (6)	15 (5)	22 (5)
	AG	104 (94)	281 (95)	385 (95)
	GG	0 (0)	0 (0)	0 (0)
	T	173 (78)	459 (78)	632 (78)
	C	49 (22)	133 (22)	182 (22)
IL-10 819 T/C	TT	297 (63)	189 (64)	486 (64)
	TC	131 (28)	81 (27)	212 (27)
	CC	43 (9	26 (8)	69 (9)

compared across the genotypes homozygous wild type, heterozygous and homozygous mutant for TNF-α -308G/A, IL-10 (-1082A/G and IL-10 (-819C/T). The Pearson correlations between cytokine levels and genetic variants were high (Table 2). Overall, all of the mutations corresponded to their cytokine levels. Percent frequency of TNF-α G* allele (87 vs. 90%) and GG genotype (77 vs. 80%) were lower in infected subjects than in uninfected individuals. Likewise TNF-α A* was higher in infected individuals (3%) than in uninfected (1%) but the difference were not statistically significant (X^2 = 1.820; p = 0.403). Both IL-10 -1082A/G alleles occurred with the same frequency in malaria and malaria negative. Both A* and G* alleles had high representation in the population with G* having 53% and A* 47%. No homozygosity for mutant allele IL-10 -1082 G* was observed in this study population. IL 10 -819 alleles and genotypes also had the same frequency in *P. falciparum* infected and uninfected subjects, however the major allele T* was three times more prevalent than the minor allele IL-10 -819C*. TNF-α -308, IL-10 -1082 and IL-10 -819 SNPs were examined using chi-square test to establish whether each was associated with susceptibility to malaria in the children. However, the distribution of genotypes did not differ significantly between malaria infected and compared to uninfected for TNF-α -308 (X^2= 1.820; p= 0.403) , IL-10 –1082 (X^2= 0.242; p= 0.623) and IL-10 -819 (X^2= 0.063; p= 0.969).

DISCUSSION

Resistance to malaria is affected by both the host and parasite factors. Host factors include genetic factors that pre-determine responses to malaria infection. These may include immune response genes and polymorphism in genes controlling acute phase responses. In this study, pro inflammatory cytokine, TNF-α and anti-inflammatory cytokine IL-10 SNPs were investigated to establish distribution of genotypes in malaria and non -malaria subjects living in area of stable *P. falciparum* transmission. SNPs were analysed against *P. falciparum* infection status, parasitaemia and cytokine levels in both groups of participants.

The parasite counts obtained and severity of infection as defined from the clinical presentation showed that the level of parasitaemia is neither related to any genetic make-up nor TNF-α or IL-10 serum levels (Table 2). This is in agreement with the results obtained from studies done in Tanzania and Gabon (Migot-Nabias et al., 2000; Mombo et al., 2003; Stirnadel et al., 1999) where lack of association between TNF-α and IL-10 genetic variants and *P. falciparum* infection were observed. The present study did not find correlations between TNF-α - 308 and parasitaemia which is in accordance with findings from Burkina Faso by Flori et al. (2005). Instead they went on to find significant correlation between TNF-α -1031T, TNF-α 1304A and TNF-α 851A and parasitaemia which is suggestive of involvement of TNF-α genetic variants in *P. falciparum* infection.

Variation was observed on TNF-α - 308 gene where 2% (6) were AA, 19.0% (75) GA and 80% (316) GG. The AA genotype predicts high production of TNF-α and the AG genotype predicts a medium level production while GG predicts low-level production of TNF-α. This was

confirmed when plasma levels of TNF-α were found to correlate well with the TNF-α -308 genotypes (Table 2). This is consistent with what was reported in other studies (Cuenca et al., 2001; Grau et al., 1989; Wilson et al., 1997). We would have expected the patients with very high levels of TNF-α, carriers of TNF-α -308 AA genotype present with severe malaria but in this study we found no relationship suggesting counteraction from other inflammatory controlling agents. Tumour necrosis factor alpha has been associated with the malaria immunopathology in that it is a pro-inflammatory cytokine and induces adhesion molecules such as ICAM-1, E-selectin and V-CAM-1 (Liz-Grana et al., 2001). May be the pathology observed in our study could have been due to other factors or other pro-inflammatory cytokine gene polymorphisms such as IL-1 or IFN-γ. Other studies carried out in Gambian children and Sri Lankans have reported strong association of TNF-α -308A with susceptibility to severe malaria (McGuire et al., 1994, 1999; Wattavidanage et al., 1999). While findings showing association have been reported in different regions, contradicting findings were also reported in Thai (Hurme et al., 1998), Sudan (Mergani et al., 2010) and Gabon (Meyer et al., 2002). Such discrepant results make it difficult to fully understand the role of TNF-α -308G/A polymorphism in malaria. The conflicting results may suggest that TNF-α -308A may not be the mutation responsible for the phenotypic variations observed in malaria but others not analysed in this study. Also TNF-α -308A may be in linkage disequilibrium with a causal mutation close to the TNF-α gene such as MHC genes or other loci on TNF-α gene. Flori et al. (2005) however failed to show linkage disequilibrium of TNF-α -308 with any of the ten other SNPs they studied on TNF-α but had previously shown linkage of mild malaria with MHC genes. The TNF-α promoter variants influence the balance of IL-10: TNF-α in the plasma, which in turn, affects the outcome in terms of clinical complications.

As with TNF-α, genotypic variation in the promoter region of IL-10 has been shown to account for the differences in the levels that are produced by an individual. IL-10 is an anti-inflammatory cytokine whose levels are critical in modulating the inflammatory response. The IL-10 gene contains three common polymorphisms (that is, -1082, -819 and -592) that have been reported to influence gene expression (Flori et al., 2005; Rees et al., 2002; Turner et al., 1997) with a two fold increase in transcriptional activity compared to the G allele. In this study, two polymorphisms -1082 and -819 were investigated and in accordance with these previous findings, the *G and *C allele carriers were demonstrated to be high and low producers of IL 10. GG and CC genotypes for -1082 and -819, respectively are associated with increased IL-10 production, while the -1082A and -819T are associated with a reduced production of IL-10. None of the participants were homozygous GG for IL-10 -1082. In our study, interleukin-10 (-1082) did not give much variation, 95% (385) of the subjects were of the genotype AG while only 5% (22) subjects had the genotype AA. The AA individuals are expected to be low producers of IL-10 (Abel et al., 1997) and more prone to severe malaria since the cytokine is required to counteract the effects of the pro-inflammatory cytokines such as TNF-α. As with TNF-α there was no evidence of IL-10 -1082 A/G association with malaria. There was also no association of IL-10 genotypes at position -819 of IL-10 gene promoter. The findings are consistent with those reported in Gambian children (Jallow et al., 2005) and Thailand (May et al., 2000; Ohashi, 2002) where lack of association between malaria susceptibility and IL-10 polymorphism was reported. In the current study, subjects that were found to be AG carriers presented with mild malaria. Individuals with AG genotype are expected to produce medium levels of IL-10 a phenomenon that was confirmed in the current study. Upon analysis of association between IL-10 genotypes and parasite density, neither -819 nor -1082 produced evidence of relationship. These results are in accordance with findings from Carpenter et al. (2009) who also investigated the host genetic control of malaria susceptibility using parasite levels and clinical episodes. The results of this study raises the possibility that susceptibility to malaria is determined by IL-10 polymorphisms that were not typed in these investigations or that the effect depends on a specific combination of SNPs and microsatellite repeats with several allelic forms, being present. In conclusion, this study did not produce evidence of genetic influence of TNF-α (-308G/A), IL-10 (-1082A/G) and IL-10 (-819C/T) on development of clinical malaria in this Zimbabwean population.

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