Polymorphisms in Interleukin-1β and Interleukin-1 Receptor Antagonist Genes and Malaria in Ghanaian Children

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We have investigated the possible associations between polymorphisms in two interleukin-1 (IL-1) genes and severity of *Plasmodium falciparum* malaria in Ghanaian children with cerebral malaria, severe anaemia or uncomplicated malaria and controls. There was no significant difference in genotype and allele frequencies in IL-1β exon 5 or interleukin-1 receptor antagonist (IL-1ra) polymorphisms between the studied groups, suggesting that the two polymorphisms may not be involved in the pathogenesis of severe malaria. When parasitaemias in uncomplicated malaria patients were evaluated, a significantly higher level of parasitaemia was observed among carriers of IL-1β A2 allele as compared with noncarriers of this allele ($P = 0.01$). The mean parasitaemia in an age-matched asymptomatic group did not reveal such associations. These data suggest that IL-1β exon 5 allele 2 may play a possible role in the clinical outcome of uncomplicated malaria.

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INTRODUCTION

Cerebral malaria and severe anaemia constitute the most severe complications of *Plasmodium falciparum* malaria, which together account for the major proportion of deaths owing to severe malaria in Africa. Although the precise mechanism underlying the pathophysiology of severe anaemia is poorly understood, it is suggested that multiple host genetic factors are involved. Cytokines including tumour necrosis factor (TNF), interleukin-1 (IL-1) and IL-10 have been implicated as important mediators of this immunopathological process [1–3]. For example, elevated concentrations of TNF have been found in African children with severe malaria [3]. This effect is suggested to be regulated by polymorphism in a locus of the TNF promoter gene [4]. With regard to IL-1, the situation is less clear. Whilst others and we have observed elevated levels of IL-1 in cerebral malaria patients, some investigators did not observe this [1, 3, 5, 6]. There are indications that IL-1 may play a role in the pathogenesis of severe malaria, as in vascular tissues, and IL-1-induced shock results in an increase in the plasma concentration of nitric oxide and increased expression of adhesion molecules implicated in the pathogenesis cerebral malaria [7].

The pro-inflammatory cytokines IL-1β and IL-1α and their receptor antagonist (IL-1ra) play a major role in initiating and modulating immune responses [7]. The genes coding for these cytokines are clustered on chromosome 2q12–22 and are polymorphic at several loci [8]. There is a considerable interindividual variation in the production of these cytokines, and several studies have presented evidence that this may be related to the existence of different alleles. Findings of elevated plasma concentrations of IL-1ra in patients with various diseases suggest that this antagonist to IL-1 is a part of the host's natural response to illnesses [7].

A bi-allelic polymorphism in the IL-1β gene in exon 5 has been described, with the allele A2 representing an IL-1β high-secretory phenotype [9]. Similarly, the A2 alleles of the

variable number of tandem repeat (VNTR) polymorphism in intron 2 of IL-1ra is associated with higher production of IL-1ra [10]. Very low frequencies of the rare allele A2 of IL-1ra intron 2 VNTR have been reported among Africans [11], and this made us hypothesize that allelic frequencies of the IL-1 genes may contribute to the severity of malaria. The aims of the present study were to analyse the frequencies of A2 allele polymorphisms of IL-1β exon 5 and VNTR of IL-1ra intron 2, and to associate these polymorphisms with the severity of disease as well as with other clinical manifestations of malaria in a case-control study of Ghanaian children.

MATERIALS AND METHODS

Study population and sampling. All children between 1 and 12 years of age, admitted to the Korle Bu Teaching Hospital in Accra, Ghana during the peak malaria season in 1998 and 1999, were screened for inclusion in the study. A total of 461 children including 86 with cerebral malaria, 55 with severe malarial anaemia, 107 with uncomplicated malaria, 102 with asymptomatic malaria and 111 controls were selected. For clinical case definition, the following inclusion criteria were used: axillary temperature of >37.5 °C and asexual *P. falciparum* parasitaemias of >2500/μl. All children with a positive sickling test (metasulphite method) and any other disease apart from malaria were excluded. Patients were divided into three clinical categories: cerebral malaria, severe anaemia and uncomplicated malaria based on the following criteria. Cerebral malaria was defined as an unarousable coma with a Blantyre coma score of 3 or less for more than 60 min and no sign of meningitis or encephalitis. Severe anaemia was defined as haemoglobin levels of <50 g/l on admission in a child with parasitaemia, with no other cause of anaemia and with full consciousness (a score of 5 on the Blantyre coma scale) [12]. Children with uncomplicated malaria had a haemoglobin of >80 g/l, a parasitaemia of >2500/μl, with full consciousness and no other complications. We also had 102 children with asymptomatic malaria who had parasitaemia of >2500/μl but nonfebrile (axillary temperature of 37.5 °C). Controls were age-matched children who presented to the same hospital with a variety of infections and noninfectious diseases unrelated to malaria. Patients and controls were enrolled in the study only following informed, signed, parental consent. The Ethical and Protocol Review Committee of the University of Ghana Medical School and the Ministry of Health approved the study.

Blood sample collection. Venous blood was drawn into sterile vacutainers containing ethylenediaminetetraacetic acid (EDTA) on the day of admission to hospital.

Parasitological examination. Thick and thin blood films stained with the Giemsa stain for the detection of plasmodium parasites were taken from all subjects including controls. The parasitaemia was quantified relative to 300 leucocytes or white blood cells (WBCs) in the thick film, and the parasite count per microlitre of blood was calculated using the measured WBC count. A slide was considered parasite-free only if no parasite had been seen after counting 1000 WBCs. A routine quality control ensured the re-examination of the blood smears, without being aware of the previously recorded result.

DNA extraction. The phenol/chloroform method with proteinase K digestion was used to extract genomic deoxyribonucleic acid (DNA) from EDTA-preserved buffy coat samples [13].

Sample typing. Polymerase chain reaction (PCR) was used to amplify DNA candidate genes of interest using a thermal cycler, GeneAmp PCR System 2400 (Perkin Elmer, Nowak, CA, USA). *IL-1ra VNTR polymorphism*: Fragments containing variable numbers of identical tandem repeat of 86 base pairs (bp) were amplified using the following oligonucleotides flanking the region as primers: 5′-CTCAGCCAACACTCCTAT-3′ and 5′-TCCTGGTCTGCAGGTAA-3′ [10]. Amplification was performed in a 20 μl reaction mix containing a final concentration of 100 ng of genomic DNA, 200 nM dNTPs (Life Technologies, Gaithersburg, MD, USA), 1× PCR reaction buffer containing 1.5 mM $MgCl_2$, 0.1 μM of each primer and 1.5 U Taq Polymerase (Applied Biosystems, Braneburg, NJ, USA) per tube. The following reaction conditions were used: three cycles at 97 °C for 30 s, 55 °C for 1 min 30 s and 74 °C for 1 min; 30 cycles at 97 °C for 30 s, 55 °C for 30 s and 74 °C for 30 s, followed by one cycle at 72 °C for 10 min and cooling at 4 °C. The PCR products of 410 bp (allele 1, four repeats of the 86 bp region), 240 bp (allele 2, two repeats), 500 bp (allele 3, five repeats), 325 bp (allele 4, three repeats) and 595 bp (allele 5, six repeats) were analysed by electrophoresis on 2% agarose gels stained with ethidium bromide. *IL-1β exon 5 restriction fragment length polymorphism*: The oligonucleotides 5′-GTTGTCATCAGACTTGACC-3′ and 5′-TTCAGTTCATATGGACCAGA-3′ were used to amplify the region which contains the *Taq*I polymorphic site within exon 5 of the IL-1β gene [14]. Amplification was performed under the following conditions: at 97 °C for 30 s, 55 °C for 1 min 30 s and 72 °C for 1 min; 30 cycles at 97 °C for 30 s, 55 °C for 30 s and 74 °C for 30 s, followed by one cycle at 72 °C for 10 min and cooling at 4 °C. After *Taq*I digestion of the 249 bp PCR product, either two fragments of 135 and 114 bp were formed (allele 1), or it remained intact at 249 bp (allele 2).

Antibody measurements. Total immunoglobulin G (IgG) and antimalarial IgG were determined as previously described [15]. Briefly, for total serum IgG, enzyme-linked immunosorbent assay plates were coated with affinity-purified goat antihuman IgG (Vector Laboratories, Burlingame, CA, USA). For determining antimalarial IgG antibodies, the plates were coated with 50 μl of Percoll-fractionated and lysed parasite antigens (10 μg/μl) per well at 4 °C overnight. The test sera were diluted to 1 : 1000. Bound IgG was assayed with biotinylated goat antihuman IgG antibodies (Vector Laboratories), followed by alkaline phosphatase (ALP)-conjugated streptavidin (Mabtech, Stockholm, Sweden).

Statistical analysis. The χ^2-analysis was used to test the comparison between gene and allele frequencies between patient groups and control. Fisher's exact tests were used when necessary. Unpaired Student's *t*-test was used to compare differences in mean parasitaemia between clinical and asymptomatic cases. A *P*-value of <0.05 was considered to be statistically significant. The analysis was performed with the computer program STATISTICA 5.1 (StatSoft, Tulsa, OK, USA).

RESULTS

IL-1β and IL-1ra genotype and allele frequencies in patients and controls

The distribution of the genotypes of IL-1β exon 5 and IL-1ra VNTR polymorphisms in cerebral malaria, severe anaemia, uncomplicated malaria and controls is shown in Table 1.

Table 1. Genotype frequencies of interleukin-1β (IL-1β) exon 5 and interleukin-1 receptor antagonist (IL-1ra) intron 2 VNTR genes in patients with cerebral malaria, severe anaemia, uncomplicated malaria and controls*

Genotype	Cerebral malaria	Severe anaemia	Uncomplicated malaria	Control
IL-1β exon 5				
A1A1	49 (62.8)	33 (60.0)	67 (62.6)	66 (63.5)
A1A2	25 (32.1)	19 (34.5)	35 (32.7)	34 (32.7)
A2A2	4 (5.1)	3 (5.5)	5 (4.7)	4 (3.8)
Total	78	55	107	104
P	0.67	0.64	0.93	0.90
IL-1ra intron 2				
A1A1	80 (93.0)	47 (94.0)	98 (94.2)	102 (91.9)
A1A2	5 (5.8)	2 (4.0)	5 (4.8)	7 (6.3)
A2A2	1 (1.2)	1 (2.0)	1 (1.0)	2 (1.8)
Total	86	50	104	111
P	0.82	0.58	0.95	0.76

*Values are the number of subjects in each group % (in parentheses). The *P*-values after comparing cases and controls between (a) IL-1β exon 5 A2A2 and A1A2, A1A1 and (b) IL-1ra intron 2 A2A2 and A1A2, A1A1 using χ^2-test (Fisher's exact test).

No significant differences in genotype frequencies in IL-1β exon 5 polymorphism were observed between any of the study groups. The allelic frequency did not reveal any differences between the studied groups (data not shown). Similarly, no differences were seen with the IL-1ra VNTR polymorphism. Because the genes for IL-1β and IL-1ra are located in the same region of chromosome 2 [8], we investigated whether the alleles of these genes associate together. There was no association between the simultaneous carriage of IL-1β allele 2 and IL-1ra allele 2 in any of the study groups (data not shown).

IL-1β and IL-1ra polymorphisms in relation to parasitaemia and serology

In order to investigate the possible consequences of the IL-1 gene polymorphisms on the outcome of uncomplicated malaria, we compared parasite densities with the genotypes or carriages of the alleles at the two IL-1 loci within the uncomplicated malaria group. As summarized in Table 2, the mean parasitaemias of carriers of IL-1β A2 allele were significantly higher than noncarriers of this allele ($P < 0.05$). The mean parasitaemia was also higher for the carriers of IL-1β A2 allele in an age-matched asymptomatic group but did not reach statistical significance. Differences in the levels between the two categories in relation to the carriage or noncarriage of IL-1β A2 were not significant. A similar comparison of mean parasitaemia for IL-1ra polymorphism did not reveal any differences (data not shown).

Table 2. Comparison of *Plasmodium falciparum* mean parasitaemia of children with uncomplicated and asymptomatic malaria, who were carriers or noncarriers of IL-1β exon 5 A2 allele*

	Carriage of IL-1β exon 5 A2 allele		
Category	Yes	No	*P*
Uncomplicated	28,042 ± 8191 (43)	12,501 ± 6942 (64)	0.01
Asymptomatic	25,294 ± 7227 (37)	16,178 ± 5061 (65)	0.09

*Values are geometric mean ± standard deviation (SD) of parasites per microlitre. Number of individuals are given in parentheses. *P*-values after unpaired Student's *t*-test.

We tested if the levels of total serum IgG and antimalarial IgG antibodies were associated with a particular genotype or allele of the two polymorphisms. Neither total IgG nor antimalarial IgG antibody levels differed in any of the study groups in these comparisons. No relationships were found between the two polymorphisms and gender or age.

DISCUSSION

Using PCR-based typing techniques, we analysed polymorphisms of the IL-1β exon 5 and IL1-ra VNTR genes in malaria patients and controls. The study did not reveal any significant differences between the study groups in genotypic or allelic frequencies of the two polymorphisms. This, together with the absence of an association between the two IL-1 loci and malaria severity, suggests that these polymorphisms are not involved in the regulation of the pathogenesis of severe malaria in the Ghanaian study population. The very low frequencies of IL-1ra allele 2 in all study groups support the previous findings in which the IL-1ra minisatellite of the intron 2 loci was not found to be associated with severe malaria in a Gambian population [11]. Although low frequencies of this allele are found among Africans, the allele on its own might not be a genetic marker of malaria severity. Indeed, information on the role of IL-1 and its antagonist in malaria susceptibility or severity is either scanty or inconclusive. We have previously reported on the elevated levels of IL-1ra in cerebral malaria patients in the same Ghanaian population [6], which is consistent with earlier reports in Gambia [1]. For IL-1β, some investigators have reported increased levels in cerebral malaria, while others have not observed such an increase [1, 3, 5].

In our study, the significantly higher parasitaemias amongst the carriers of IL-1β A2 than amongst noncarriers in uncomplicated malaria patients suggest a possible role for IL-1β polymorphism in the clinical outcome of uncomplicated malarial infections. IL-1 has been reported to induce fever, which may retard the development of *P. falciparum* parasites [1].

This might indicate an antiparasitic role of IL-1 in malaria. However, other factors are likely to be involved, as we did not observe any significant differences in parasitaemia between the uncomplicated and asymptomatic malaria cases.

The IL-1β and IL-1ra genes are closely related on the long arm of chromosome 2 and seem to exert their effect in association [8]. One study of inflammatory diseases has shown that IL-1ra allele 2 when in linkage with IL-1β exon 5 allele 2 is associated with disease severity, whereas the individual alleles did not have any effect [14]. The lack of relationship between the IL-1 genes and malaria severity in our study does not rule out their possible involvement through association with other cytokine genes. IL-1 belongs to a group of cytokines with overlapping biological properties [7]. In addition to its suggested pyrogenic effect on parasites, IL-1 may play a role in the pathogenesis of severe malaria. A previous study reported high serum levels of IL-1α preferentially in patients who developed severe malaria [3]. IL-1 is known to synergize with other cytokines, notably TNF which is implicated in the pathogenesis of severe malaria. In CBA mice infected with *Plasmodium vinckei*, a low level of IL-1β or IL-1α when administered together with TNF increased the level of plasma-reactive nitrogen intermediates and caused hypoglycaemia – the two factors implicated in the pathogenesis of cerebral malaria. These effects were, however, negligible when the cytokines were administered alone, pointing to the fact that both the cytokines are required for such an effect [16]. With the functional significance of IL-1 in the pathogenesis of severe malaria being controversial, it is worthwhile investigating the possible association of polymorphisms in the IL-1 gene with that of the TNF gene, especially the TNF-308 loci which have been shown to be associated with severe malaria [4]. This will be an effective way of assessing the role of IL-1 gene polymorphism in malaria severity.

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REFERENCES

1 Jakobsen PH, McKay V, Morris-Jones SD *et al*. Increased concentrations of interleukin-6 and interleukin-1 receptor antagonist and decreased concentrations of beta-2-glycoprotein I in Gambian children with cerebral malaria. Infect Immun 1994;62:4374–9.

2 Kurtzhals JA, Adabayeri V, Quarm-Goka B *et al*. Low plasma concentrations of interleukin 10 in severe malarial anaemia compared with cerebral and uncomplicated malaria. Lancet 1998;351:1768–72.

3 Kwiatkowski D, Hill AV, Sambou I *et al*. TNF concentration in fatal cerebral, non-fatal cerebral, and uncomplicated *Plasmodium falciparum* malaria. Lancet 1990;336:1201–4.

4 McGuire W, Knight JC, Hill AV, Allsopp CE, Greenwood BM, Kwiatkowski D. Severe malarial anemia and cerebral malaria are associated with different tumor necrosis factor promoter alleles. J Infect Dis 1999;179:287–90.

5 Molyneux EM, Taylor TE, Wirima JJ, Borgstein A. Tumor necrosis factor, interleukin-6, and malaria. Lancet 1991;337:1098.

6 Akanmori BD, Kurtzhals JA, Goka BQ *et al*. Distinct patterns of cytokine regulation in discrete clinical forms of *Plasmodium falciparum* malaria. Eur Cytokine Netw 2000;11:113–8.

7 Dinarello C. Biologic basis for interleukin-1 in disease. Blood 1996;87:2095–147.

8 Steinkasserer A, Spurr NK, Cox S, Jeggo P, Sim RB. The human IL-1 receptor antagonist gene (IL1RN) maps to chromosome 2q14–q21, in the region of the IL-1 alpha and IL-1 beta loci. Genomics 1992;13:654–7.

9 Pociot F, Molvig J, Wogensen L, Worsaae H, Nerup J. A TaqI polymorphism in the human interleukin-1 beta (IL-1 beta) gene correlates with IL-1 beta secretion in vitro. Eur J Clin Invest 1992;22:396–402.

10 Tarlow JK, Blakemore AI, Lennard A *et al*. Polymorphism in human IL-1 receptor antagonist gene intron 2 is caused by variable numbers of an 86-bp tandem repeat. Hum Genet 1993;91:403–4.

11 Bellamy R, Kwiatkowski D, Hill AV. Absence of an association between intercellular adhesion molecule 1, complement receptor 1 and interleukin 1 receptor antagonist gene polymorphisms and severe malaria in a West African population. Trans R Soc Trop Med Hyg 1998;92:312–6.

12 Molyneux ME, Taylor TE, Wirima JJ, Borgstein A. Clinical features and prognostic indicators in paediatric cerebral malaria: a study of 131 comatose Malawian children. Q J Med 1989;71: 441–59.

13 Marmur J. A procedure for the isolation of deoxyribonucleic acid from microorganisms. J Mol Biol 1961;3:208–18.

14 Bioque G, Crusius JB, Koutroubakis I *et al*. Allelic polymorphism in IL-1 beta and IL-1 receptor antagonist (IL-1Ra) genes in inflammatory bowel disease. Clin Exp Immunol 1995;102: 379–83.

15 Perlmann H, Helmby H, Hagstedt M *et al*. IgE elevation and IgE anti-malarial antibodies in *Plasmodium falciparum* malaria: association of high IgE levels with cerebral malaria. Clin Exp Immunol 1994;97:284–92.

16 Rockett KA, Awburn MM, Rockett EJ, Clark IA. Tumor necrosis factor and interleukin-1 synergy in the context of malaria pathology. Am J Trop Med Hyg 1994;50:735–42.