Allelic polymorphisms in the repeat and promoter regions of the interleukin-4 gene and malaria severity in Ghanaian children

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SUMMARY

Immunoglobulin E has been associated with severe malaria suggesting a regulatory role for interleukin (IL)-4 and/or IgE in the pathogenesis of severe malaria. We have investigated possible associations between polymorphisms in the IL-4 repeat region (intron 3) and promoter regions (IL-4 +33CT and −590CT) in Ghanaian children with severe malaria. There was a significantly higher frequency of IL-4 intron-3 B1B1 genotype in the cerebral malaria group \( P < 0.0001, \text{odds ratio (OR)} = 8.7 \). The genotype and allele frequencies of the IL-4 −590 and +33 polymorphisms did not differ between the four study groups. Carriers of IL-4 +33T/−590T with cerebral malaria had elevated total IgE compared to non-carriers \( P = 0.03 \). Our data suggest that IL-4 and/or IgE play a regulatory role in the pathogenesis of severe or complicated malaria.

Keywords falciparum IgE IL-4 polymorphism pathogenesis Plasmodium severe malaria

INTRODUCTION

Plasmodium falciparum malaria infections in humans display a remarkable range of disease severity, from asymptomatic to severe disease, including severe anaemia, cerebral malaria and eventually death. Approximately 1–2% of clinical attacks of malaria in African children are life-threatening [1]. Although the reasons for these differences in susceptibility to the disease are multi-factorial, data are accumulating that they can be modulated by host genetic factors.

A number of host genes have been identified which seem to contribute to susceptibility and/or protection against severe malaria. These include haemoglobinopathies and other red blood cell mutations [2], as well as polymorphisms in genes encoding for the major histocompatibility complex (MHC) class I [3]. Candidate genes regulating the production or expression of the inflammatory cytokine tumour necrosis factor (TNF) [4,5], the intercellular adhesion molecule-1 (ICAM-1) [6,7], the inducible nitric oxide synthase [8] and the mannose-binding lectin [9] have also been implicated. Recently, an association between CD36 deficiency and an increased risk of severe malaria has been reported [10].

In a recent candidate-region approach a sib-pair linkage between the chromosome region 5q31-q33 and P. falciparum blood infection levels was reported [11,12]. This region contains numerous candidate genes encoding immunologically important molecules such as cytokines, growth factors and growth factor receptors, all involved in the control of immunity to P. falciparum blood stage infections [13]. The region is also linked to elevated serum levels of immunoglobulin E (IgE) [14] and protection against schistosomiasis [15]. This is of great interest, because we and others have recently reported a possible regulatory role of antimalarial IgE in the pathogenesis of P. falciparum malaria [16,17].

The interleukin (IL)-4 gene is pleiotropic, located in the 5q31–q33 region, with multiple immune-modulating functions on a variety of cell types [18]. IL-4 serves as an important regulator in isotype switching from IgM/IgG to IgE [19,20]. It also regulates the differentiation of precursor T helper-cells into the Th2 subset that regulates humoral immunity and specific-antibody production [21]. In the human P. falciparum system IL-4 has been shown to be involved in the regulation of antimalarial antibody responses, including antimalarial IgE [22,23].
Several polymorphisms in the IL-4 gene have been described, four of which are located in the promoter region of the gene [24]. Some of these polymorphisms have been implicated in the regulation of total IgE production [14,25].

The aim of this study was to analyse three known IL-4 polymorphisms, namely, a single nucleotide polymorphism (SNP) in the IL-4 promoter region (C → T) at position −590 base pairs from the open reading frame, one SNP at position + 33 relative to the transcription initiation site and the variable number of tandem repeat (VNTR) region in intron 3 of the IL-4 gene.

The study was conducted at the Korle-Bu Teaching Hospital in Accra, Ghana during the malaria transmission season in 1998 and 1999 (June–September). Children aged between 0·5 and 12 years were studied. The general inclusion criteria were axillary temperature of >37·5°C and asexual P. falciparum parasitaemia of >2500/µl. All children with a positive sickling test (metabisulphite method) and any other disease apart from malaria were excluded.

The malaria patients were divided into three clinical categories: cerebral malaria, severe anaemia and uncomplicated malaria based on the following criteria. For cerebral malaria, the inclusion criteria were asexual P. falciparum parasitaemia, unarousable coma, with a score of 3 or less on the Blantyre coma scale [26] for more than 60 min and no sign of meningitis or encephalitis. For severe anaemia the inclusion criteria were asexual parasitaemia of >5 g/dl, no other cause of anaemia and full consciousness (score of 5) for uncomplicated malaria in children with cerebral malaria, severe anaemia, uncomplicated malaria or controls and to see if any of the polymorphisms were correlated with severity of disease and total IgE and antibody levels.

MATERIALS AND METHODS

Study population and sampling

The study was conducted at the Korle-Bu Teaching Hospital in Accra, Ghana during the malaria transmission season in 1998 and 1999 (June–September). Children aged between 0·5 and 12 years were studied. The general inclusion criteria were axillary temperature of >37·5°C and asexual P. falciparum parasitaemia of >2500/µl. All children with a positive sickling test (metabisulphite method) and any other disease apart from malaria were excluded.

The malaria patients were divided into three clinical categories: cerebral malaria, severe anaemia and uncomplicated malaria based on the following criteria. For cerebral malaria, the inclusion criteria were asexual P. falciparum parasitaemia, unarousable coma, with a score of 3 or less on the Blantyre coma scale [26] for more than 60 min and no sign of meningitis or encephalitis. For severe anaemia the inclusion criteria were asexual P. falciparum parasitaemia, haemoglobin levels <5 g/dl, no other cause of anaemia and full consciousness (score of 5) for uncomplicated malaria, as for severe anaemia but with haemoglobin >8 g/dl, a parasitaemia of >2500/µl and no other complications. The helminthic status of patients was not available. Controls were children of the same age range as cases who presented to the same hospital with relatively mild illness requiring out-patient treatment but negative for plasmodium parasites. The majority of these children had gastroenteritis, upper respiratory tract infections or meningitis. Thick and thin blood films stained with Giemsa stain for detection of plasmodium parasites was taken from all subjects, i.e. cases and controls. Patients and controls selected for the study were from different ethnic groups in Ghana who enrolled at the hospital. The clinical characteristics of the patients and controls are shown in Table 1.

Blood collection

Venous blood was drawn into vacutainers containing heparin or ethylene diamine tetra-acetic acid (EDTA) and plasma was obtained by centrifugation at 4°C within 30 min of blood collection and stored at −80°C. Buffy coats were collected and stored at −80°C.

DNA extraction

The standard protein K digestion and phenol/chloroform method was used to extract genomic DNA from EDTA preserved buffy coat samples [27].

Typing for cytokine allelic polymorphism

VNTR polymorphism in intron 3 of IL-4 gene. The region that contains the VNTR polymorphisms within the IL-4 intron 3 region was amplified by a polymerase chain reaction (PCR). The oligonucleotides 5’-TAGGCTGAAAGGGGGAACG-3’ and 5’-CTGTTACCTCAACTGCTCC-3’ flanking this region were used as primers. Amplification was performed in a 20 µl reaction mix containing 1 µl of genomic DNA, 200 nm dNTPs (Life Technologies, Gaithersburg MD, USA), 0·1 µM of each primer and 1·5 U Taq polymerase (Applied Biosystems, Braneburg, NJ, USA) per tube and using a programmed thermal cycler Gene Amp PCR System 2400 (Perkin Elmer, Norwalk CA, USA). The following reaction conditions were used: 95°C for 10 min; 32 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 30 s followed by one cycle of 72°C for 5 min. The PCR products were analysed directly by electrophoresis on 2% agarose gels stained with ethidium bromide. Alleles of 183 and 253 base pairs (bp) in lengths were recognized as allele B1 and B2, respectively.

IL-4 −590 and IL-4 +33 polymorphisms. Primers used for amplification of IL-4 −590 were 5’-ACTAGCCCTCACTGATAC-G-3’ and 5’-GTTGTAATGCAGTCCTCCTG-3’ and for IL-4 + 33 5’-GTGCTGATTTGCCCACAAGTGACTG-3’ and 5’-TGGACTGCCAAACCACCGT-3’. The PCR conditions were 95°C for 10 min followed by 30 cycles of 95°C for 50 s, 62°C for 50 s, 72°C for 50 s followed by one cycle of 72°C for 5 min.

Table 1. Clinical characteristics and antibody levels of patients and controls

<table>
<thead>
<tr>
<th></th>
<th>Cerebral malaria</th>
<th>Severe anaemia</th>
<th>Uncomplic. malaria</th>
<th>Control</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of subjects</td>
<td>112</td>
<td>90</td>
<td>140</td>
<td>134</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>4·4</td>
<td>4·1</td>
<td>4·5</td>
<td>3·9</td>
<td></td>
</tr>
<tr>
<td>Hb concentration (g/dl)</td>
<td>7·4</td>
<td>2·4</td>
<td>8·8</td>
<td>9·1</td>
<td></td>
</tr>
<tr>
<td>Parasitaemia (µl)</td>
<td>28·789</td>
<td>20·923</td>
<td>33·120</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total serum IgE (ng/ml)</td>
<td>111·10</td>
<td>98·01</td>
<td>72·41</td>
<td>77·18</td>
<td>0·03</td>
</tr>
<tr>
<td>(33·3)</td>
<td>(29·1)</td>
<td>(36·5)</td>
<td>(27·8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-malarial IgE (ng/ml)</td>
<td>0·71</td>
<td>0·65</td>
<td>0·62</td>
<td>0·59</td>
<td>0·09</td>
</tr>
<tr>
<td>(0·26)</td>
<td>(0·19)</td>
<td>(0·10)</td>
<td>(0·20)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-malarial IgG (µg/ml)</td>
<td>11·32</td>
<td>10·94</td>
<td>9·98</td>
<td>9·80</td>
<td>0·07</td>
</tr>
<tr>
<td>(5·4)</td>
<td>(4·5)</td>
<td>(4·6)</td>
<td>(4·1)</td>
<td></td>
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</tr>
</tbody>
</table>

*Mean; †geometric mean with standard errors (SE) in brackets; ‡comparing all groups using the Kruskal–Wallis ANOVA test.

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For IL-4 –590, the reaction conditions were the same as for IL-4 intron 3. To detect IL-4 –590 and IL-4 +33 polymorphisms, PCR products were digested further with the restriction enzymes BsmFI and BsmAI (New England Biolabs Inc., USA), respectively, under conditions described by Walley et al. 1996 [28]. For IL-4 –590 this resulted in two fragments of 192 bp and 60 bp for the –590C allele and an intact (252 bp) product for the –590T allele. For IL-4 +33 polymorphism, 190 bp for +33C allele and 150 bp and 38 bp for +33T allele were identified after enzyme digestion.

**Antibody measurements**

Total IgE and antimalarial specific IgE were determined as described previously [29]. Briefly, for total serum IgE enzyme-linked immunosorbent assay (ELISA) plates were coated with affinity purified goat antihuman IgE (Vector Laboratories, CA, USA). Anti-malarial IgG was detected using streptavidin–alkaline phosphatase (ALP)-conjugated streptavidin and IgE antibodies (Vector Laboratories, CA, USA) followed by anti-IgG antibody. Bound IgE was assayed with biotinylated goat anti-human antibody. There were dilutions 1:1000 for total IgE and 1:50 for antimalarial IgE antibodies. There were no significant differences in total IgE levels between study groups.

**Statistical analysis**

The Sigma STAT 2.0 statistics package was used for the statistical analysis to calculate \( \chi^2 \) values with Yates’s correction. Associations were deemed significant if a P-value was equal to or less than 0.05. Test for linkage disequilibrium of polymorphisms was obtained by determining \( \chi^2 \) values. Allelic distribution was tested according to the Hardy–Weinberg equilibrium. A comparison between cases and controls of the -590 and +33 loci revealed a significantly higher frequency of the B1 allele than the B2 allele (P = 0.006, OR = 2.04). For the IL-4 –590 and +33, no differences were seen in the genotype or allele frequency distribution of these polymorphisms between any of the study groups.

**Association of genes and alleles of IL-4 gene polymorphisms**

We investigated the possible linkage disequilibrium between polymorphisms at the three IL-4 loci within the total study material. There was a significant association between IL-4 intron 3 and +33 loci (P = 0.009) and between the IL-4 +33 and –590 loci (P = 0.003). Based on this association, we grouped individuals into simultaneous carriage of two alleles at the three loci. There was a significant higher frequency of the IL-4 +33T/-590T allele association in cerebral malaria patients (P = 0.004, OR = 1.45) which was not seen in the other groups (P = 0.57, OR = 1.32 for severe malaria and the P = 0.88 for uncomplicated malaria).

**RESULTS**

**IL-4 genotypes and allele frequencies in patients and controls**

A total of 112 patients with cerebral malaria (mean age = 4.4), 90 with severe anaemia (age = 2.4), 140 with uncomplicated malaria (mean age = 4.5) and 134 controls (mean age = 3.9) were included in this study (Table 1). Table 2 summarizes the distribution of genotypes and allelic frequencies of the three IL-4 polymorphisms among the patients and controls. Genotype distributions of the polymorphisms were in agreement with the Hardy–Weinberg equilibrium. A comparison between cases and controls of the B1B1 genotype with the rest (B1B2 and B2B2) revealed a significantly higher number of the B1B1 genotype for cerebral malaria (P < 0.001, OR = 8.7) but not for severe anaemia or uncomplicated malaria groups. Similarly, the allelic frequencies of the intron 3 polymorphism also revealed a significantly higher frequency of the B1 allele than the B2 allele (P = 0.006, OR = 2.04). For the IL-4 –590 and +33, no differences were seen in the genotype or allele frequency distribution of these polymorphisms between any of the study groups.

**Table 2. Distribution of IL-4 genotypes in patient groups with cerebral malaria, severe anaemia, uncomplicated malaria and control subjects**

<table>
<thead>
<tr>
<th></th>
<th>IL-4 intron 3</th>
<th>IL-4 –590</th>
<th>IL-4 +33</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebral</td>
<td>18 (17.3)</td>
<td>44 (42.3)</td>
<td>42 (40.4)</td>
</tr>
<tr>
<td>Malaria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Severe</td>
<td>5 (9.1)</td>
<td>27 (50.0)</td>
<td>23 (41.9)</td>
</tr>
<tr>
<td>Anaemia</td>
<td>10 (8.2)</td>
<td>48 (39.3)</td>
<td>64 (52.5)</td>
</tr>
<tr>
<td>Uncomp.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malaria</td>
<td>3 (2.3)</td>
<td>54 (42.3)</td>
<td>71 (55.5)</td>
</tr>
<tr>
<td>Control</td>
<td>36</td>
<td>173</td>
<td>200</td>
</tr>
</tbody>
</table>

Values are the number of subjects in each group; % in parenthesis. Comparing cases and controls between homozygotes for IL-4 intron 3 B1B1 and B2B2 after \( 2 \times 2 \chi^2 \) test gave the P-values: \( \chi^2 = 14.9, P < 0.0001 \) for cerebral malaria, \( \chi^2 = 3.6, P = 0.06 \) for severe anaemia and \( \chi^2 = 3.04, P = 0.08 \) for uncomplicated malaria.

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the study categories (data not shown). We also looked at associations between carriage of the IL-4 intron 3 B1+/33, +33T/−590T or intron-3 B1/+33T alleles and total IgE. There was a significantly higher total IgE level for carriers than non-carriers of +33T/−590T alleles in the cerebral malaria group but not in the other groups (Table 3). The intron 3 B1/+33T or intron 3 B1/−590T alleles did not reveal any association. No association was found between the IL-4 polymorphisms and antimalarial IgG, IgE antibodies or parasitaemias (data not shown).

**DISCUSSION**

In this study we demonstrate a higher frequency of the B1B1 genotype in intron 3 of the IL-4 gene in a group of children with cerebral malaria compared to children with severe anaemia, uncomplicated malaria or controls. The allele was associated with cerebral malaria but not with IgE. The functional role of the B1 allele, located in the intron 3 of the IL-4 gene, is not known [30] but the polymorphism has been reported to be associated with late onset of multiple sclerosis [31] and to protect against severe joint destruction in chronic polyarthritis [32]. There are no data on the association between IL-4 intron 3 polymorphisms and IgE. Some reports in asthma where association of IL-4 gene polymorphisms with disease were reported failed to show an association of such polymorphisms with IgE [33].

Immunoglobin-class switching from IgM/G to IgE requires IL4 and/or IL-13 [19,20]. The genes encoding IL-4 are located in the 5q31–q33 region, suggesting an important regulatory role of IL-4 in the production of IgE. Several polymorphisms in the IL-4 genes have been implicated in the regulation of total IgE production. Thus, the SNP-C → T transition at position −590 bp from the open reading frame has been shown to increase promoter activity of IL-4 in a luciferase reporter-gene construct, suggesting that this mutation increases the expression of IL-4 in humans [34]. The SNP at position +33 relative to the transcription initiation site has also been found to enhance promoter activity of the gene and to be associated with atopic diseases [14,25]. In our study material the −590 and +33 genotypes and alleles did not differ between the study groups. SNPs have many advantages in the dissection of diseases due to the fact that they are found throughout the genome and at high frequency. An association between the +33 and −590 loci as well as the simultaneous carriage of +33T/−590T with cerebral malaria were seen in our study. Suzuki et al. have reported of strong linkage disequilibrium between the −590T allele and the +33T allele in a Japanese population [35]. Thus, both alleles may enhance transcription and co-operate in the regulation of production of IgE.

**Table 3.** Concentration of total IgE of carriers and non-carriers of IL-4 −590T/+33T alleles among cerebral malaria, severe anaemia, uncomplicated malaria patients and controls

<table>
<thead>
<tr>
<th>Carriers of IL-4 +33T/−590T</th>
<th>Severe anaemia</th>
<th>Uncomp. malaria</th>
<th>Control malaria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebral malaria</td>
<td>150.0 (39.9)</td>
<td>72.3 (36.6)</td>
<td>61.9 (32.4)</td>
</tr>
<tr>
<td>Uncomp. malaria</td>
<td>114.2 (36.7)</td>
<td>66.0 (40.2)</td>
<td>91.0 (43.6)</td>
</tr>
<tr>
<td>Non-carriers of IL-4 +33T/−590T</td>
<td>0.03</td>
<td>0.19</td>
<td>0.80</td>
</tr>
</tbody>
</table>

Values are expressed as geometric means (SE) of concentrations ng/ml. P-values are comparison using Kruskal–Wallis ANOVA test by ranks.

Immunoglobulins E, in the form of immune complexes, have been shown to be potent inducers of TNF [36]. Overproduction of TNF has been implicated as an important factor in the pathogenesis of severe malaria [37]. In line with these data, we found that children with cerebral malaria compared to the other patient groups or controls had elevated levels of total serum IgE (Table 1). The elevated levels of total IgE in relation to IL-4 +33T/−590T carriage were seen in the cerebral malaria group only but not the other groups. The reason for this is not clear, but might indicate distinct regulatory pathways in the different groups. No differences were seen between carriers and non-carriers of the different alleles and antimalarial IgE antibodies. This might suggest that specific and total IgE production are regulated differently [36] or that our levels were too low to be able to reach statistical significance with this sample size.

The IL-4 −524T allele, which is the same as the −590T allele when counted from the ATG codon, has been shown to be associated with higher malaria antibody levels and protection against malaria in one sympatric tribe living in Burkina Faso. The same polymorphism was shown to be present in two other West African tribes, although at lower frequencies. In these latter tribes, the polymorphism was neither associated with higher antimalarial antibody levels nor protection [38]. Recently, this polymorphism in both heterozygotes and homozygotes was shown to be associated with elevated levels of total IgE in children with severe malaria (Frederica et al. unpublished data). The reason why we did not see any association between × 590T allele and elevated IgE levels might suggest that other factors are needed to amplify the effect of this IL-4 polymorphism. Regulatory elements might differ in different ethnic groups, as suggested by the findings that the association between total IgE and the −590T seem to vary depending on the genetic background of the study populations [25,28,33,39,40]. One of the mechanism(s) behind the pathogenesis of cerebral malaria involves sequestration of parasites in the endothelium of microvessels as a result of up-regulated TNF [37]. However, IL-4 is also reported to induce the close expression of the adhesion molecule V-CAM on endothelial cells [41], a receptor for the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) implicated in pathogenesis of severe malaria. Thus, it is clear those severe complications of malaria are multi-factorial and that the pathogenesis may be regulated by many different mechanisms.

Taken together, the significant association between IL-4 intron 3 polymorphism and cerebral malaria and also carriage of the IL-4 +33T/−590T alleles with cerebral malaria and high plasma levels of total IgE support our previous findings that IL-4 and/or IgE could play a regulatory role in the pathogenesis of cerebral malaria but not with IgE. The functional role of the B1 allele, located in the intron 3 of the IL-4 gene, is not known [30] but the polymorphism has been reported to be associated with late onset of multiple sclerosis [31] and to protect against severe joint destruction in chronic polyarthritis [32]. There are no data on the association between IL-4 intron 3 polymorphisms and IgE. Some reports in asthma where association of IL-4 gene polymorphisms with disease were reported failed to show an association of such polymorphisms with IgE [33].
complicated malaria. However, there is a need for further investigation of these polymorphisms in a larger sample size to confirm their usefulness as genetic markers in malaria. Infection with other parasites such as helminths, which is prevalent among African children, is known to induce IgE elevation. We did not investigate the presence or absence of helminths in our study group. However, our data and an earlier report [29] demonstrate the importance of plasmodial infection for IgE elevation with higher levels of IgE, but not of IgG, in children with cerebral malaria compared with patients with uncomplicated disease. Nevertheless, in assessing a genetic role for IL-4 in the pathogenesis of malaria it would be essential to exclude or reduce the effect of concomitant infections including helminths as well as the effect of ethnicity.

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