Severe Malarial Anemia and Cerebral Malaria Are Associated with Different Tumor Necrosis Factor Promoter Alleles

William McGuire, Julian C. Knight, Adrian V. S. Hill, Catherine E. M. Allsopp, Brian M. Greenwood, and Dominic Kwiatkowski

Experimental evidence implicates tumor necrosis factor (TNF) in the pathogenesis of malarial anemia, but there are few data relating to this hypothesis. This study found that severely anemic children with Plasmodium falciparum infection have low plasma TNF levels, in contrast to the high levels found in cerebral malaria. A previous case-control study in The Gambia found cerebral malaria, but not severe malarial anemia, was associated with the TNF \( \text{TNF}_{308} \) A allele. This study found that in the same population, severe malarial anemia was associated with the TNF \( \text{TNF}_{238} \) A allele, with an odds ratio of 2.5 (\( P < .001 \)) after stratification for HLA type. These findings suggest that severe malarial anemia and cerebral malaria are influenced by separate genetic factors situated near the TNF gene.

Around a half-million African children die each year as a consequence of severe malarial anemia. However, although most children in sub-Saharan Africa are repeatedly infected with Plasmodium falciparum, life-threatening anemia develops in only a minority. It is unclear which factors predispose these children to become markedly more anemic than others [1].

The pathogenic process of malarial anemia is multifactorial and is only partly explained by the direct destruction of erythrocytes by parasites [2]. Previous studies in The Gambia have indicated that suppression of erythropoiesis may be of additional importance. Many children with severe anemia were found to have a depressed reticulocyte response, and a significant proportion of children had gross morphologic abnormalities of erythropoietic cells in the bone marrow [3]. Several lines of experimental evidence show that erythropoiesis can be severely disrupted by inflammatory mediators such as tumor necrosis factor (TNF). In vitro, TNF suppresses proliferation of erythroid progenitor cells in human marrow cultures [4]. In vivo, erythropoiesis is inhibited by chronic exposure to TNF, achieved by implanting nude mice with TNF-secreting CHO cells [5]. TNF has been specifically incriminated as a cause of dyserythropoietic anemia in experimental malaria in mice, which recover after the administration of anti-TNF antibodies [6, 7]. TNF can also promote erythrophagocytosis, as shown by a transgenic murine model in which TNF was constitutively overexpressed [8].

In a large case-control study of severe malaria in Gambian children, the HLA class I allele, HLA-B53, and the class II allele, HLA-DRB1*1302, were shown to be associated with protection from severe malarial anemia [9]. Given that these alleles are located 0.25 Mb telomeric and 1 Mb centromeric of the TNF gene, respectively, and in view of the experimental evidence that TNF may play a role in the pathogenesis of malarial anemia, we examined the relationship of severe anemia to plasma TNF levels and to two TNF promoter alleles in the same population of Gambian children.

Materials and Methods

Index patients and control groups. Children up to 10 years old were enrolled into a case-control study of severe malaria that was based at the Royal Victoria Hospital, Banjul, and the Medical Research Council Clinic, Fajara, in The Gambia, between 1988 and 1990. Severe malarial anemia was defined by a hemoglobin concentration of <5 g/dL in association with asexual \( P. falciparum \) parasitemia of \( \geq 2500/\mu L \). Cerebral malaria was defined by a Bian-tyre coma score of \( < 3 \) (persisting for \( > 30 \) min after effective treatment of hypoglycemia or convulsions) or repeated prolonged seizures (\( > 30 \) min) in a child with \( P. falciparum \) parasitemia and no other apparent cause of fits or coma.

The severe malaria group was matched to the 2 control groups for area of residence around Banjul. The primary control group comprised children who were being treated as outpatients for minor illnesses other than malaria, predominantly respiratory and gastrointestinal infections (“mild nonmalaria” control group). The second group was children with an uncomplicated febrile illness associated with asexual \( P. falciparum \) parasitemia (“mild malaria” control group). Further details of the case-control study are reported elsewhere [9–11].

Laboratory analysis. Plasma TNF levels were measured by ELISA, and the TNF promoter TNF

\( \text{TNF}_{308} \) genotype was determined...
by polymerase chain reaction (PCR) and allele-specific oligonucleotide probing, both as previously described [10, 11].

The more recently defined TNF promoter variant, a G→A transition at position −238 nucleotides relative to the transcriptional start site [12], was typed by use of an amplification refractory mutation system PCR using a conserved primer 5'-CCGGATCATGCTTTCAGTGCC-3' together with sequence-specific primers for either the TNF−238 A allele (5'-AGACCCCCCTCGGAAATCG-3') or the TNF−238 G allele (5'-AAGACCCCCCTCGGAATCA-3') to generate 459- and 460-bp products, respectively. As a positive control, each reaction mixture contained the primers 5'-GATTCAGGTTTACTCACG-3' and 5'-CCTGAGGCCTCAAGCCT-3' to amplify a 266-bp fragment of the β2-microglobulin gene. Each 25-μL reaction mixture contained ~100 ng of genomic DNA, 67 mM Tris-HCl, 16 mM (NH₄)₂SO₄, 2 mM MgCl₂, 100 μM each dNTP (Boehringer Mannheim, Mannheim, Germany), 0.01% Tween-20, 0.5 U of Taq DNA polymerase (Bioline, London), 0.1 μM each TNF primer, and 0.2 μM each β2-microglobulin primer. The mixture was incubated at 95°C for 10 min, followed by 5 cycles of 95°C for 1 min, 67°C for 1 min, 72°C for 1 min, then 25 cycles of 95°C for 1 min, 62°C for 1 min, 72°C for 1 min, and then a final 10 min at 72°C. The products were resolved in 2% agarose gel, stained with ethidium bromide, and visualized under UV light. As part of a technical comparison of DNA typing methods, the alleles were also typed by PCR ELISA (unpublished data).

Direct DNA sequencing of the PCR product was used to confirm the validity of the typing methods. Following purification through a microcon 100 column (Amicon, Beverly, MA), PCR-amplified genomic DNA was cycle sequenced using the ABI PRISM dye terminator kit (Perkin-Elmer, Norwalk, CT) using primer 5'-CTTGAGGCCCTCAAGCCT-3' and analyzed on an ABI377 automated sequencer.

HLA types were initially determined serologically (for class I) or by restriction fragment length polymorphism using TaqI digestion with DRB- and DQB-specific probes (for class II). Further analysis of HLA types was done by allele-specific oligonucleotide hybridization of PCR products following generic amplification of the HLA-A, -B, and -C regions or the HLA-D region.

**Statistical analysis.** Continuous data were compared by the Mann-Whitney U test. Clinical groups were otherwise compared by the χ² test, using stratified Mantel-Haenszel analysis to correct for the possible confounding effects of ethnic heterogeneity and linkage with HLA class I and class II alleles.

Table 1. Clinical and laboratory features of Gambian children presenting with malaria of different severity.

<table>
<thead>
<tr>
<th></th>
<th>Mild malaria (n = 191)</th>
<th>Cerebral malaria alone (n = 166)</th>
<th>Severe malarial anemia alone (n = 63)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin concentration (g/dL)</td>
<td>8.7 (8.4–9.0)</td>
<td>8.3 (8.0–8.6)</td>
<td>4.3 (4.0–4.6)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>4.2 (4.0–4.4)</td>
<td>4.1 (3.9–4.3)</td>
<td>2.2 (2.0–2.4)</td>
</tr>
<tr>
<td>Duration of symptoms (days)</td>
<td>3.7 (3.3–4.1)</td>
<td>2.7 (2.4–3.0)</td>
<td>5.0 (4.1–5.9)</td>
</tr>
<tr>
<td>Spleen size &gt;2 cm (% of cases)</td>
<td>11</td>
<td>14</td>
<td>32</td>
</tr>
<tr>
<td>Parasitemia (×10⁷/µL)</td>
<td>59 (47–73)</td>
<td>64 (44–93)</td>
<td>30 (17–54)</td>
</tr>
<tr>
<td>TNF (pg/mL)</td>
<td>18 (15–21)</td>
<td>57 (45–78)</td>
<td>13 (8–20)</td>
</tr>
</tbody>
</table>

NOTE. Continuous data are presented as mean or geometric mean for parasitemia (95% confidence intervals).

**Results.**

Table 1 compares the clinical profile and plasma TNF levels of children with severe malarial anemia, cerebral malaria, or mild malaria. Data are presented for a subset of the total study population because plasma TNF was measured only for subjects from whom a blood sample was taken immediately after admission to the hospital. We found no significant difference in the plasma TNF levels in children presenting with severe malarial anemia compared with those with mild malaria. This contrasts with our previous finding of elevated plasma TNF levels in Gambian children with cerebral malaria [10].

With regard to the presenting features of children in each clinical category, for simplicity, children who satisfied the case definition for both cerebral malaria and severe malarial anemia are omitted from the comparison (table 1). The median age of children with severe malarial anemia was lower than that of children with cerebral malaria (P < .001) or mild malaria (P < .001). The severely anemic group reported a significantly longer duration of symptoms than children with uncomplicated malarial fever (P < .001) or cerebral malaria (P < .001). A larger proportion of children with severe malarial anemia were found to have enlarged spleens (P < .001), and the geometric mean parasitemia level on presentation was lower in the anemic group compared with those with mild malaria (P < .001).

The TNF−238 A allele was found in Hardy-Weinberg equilibrium at a frequency of ~0.06 in both of the control groups of Gambian children. We found an excess of the TNF−238 A allele in children with severe malarial anemia compared with that in either the nonmalaria control group (odds ratio [OR] = 1.7; 95% confidence interval [CI] = 1.02–2.8; P = .04, Mantel-Haenszel χ² weighted for ethnic group) or the mild malaria control group (OR = 1.9; 95% CI = 1.1–3.1; P = .02) (table 2). We found no association of the TNF−238 A allele with cerebral malaria, in contrast to our previous finding for the TNF−308 A allele, in which we reported an association of the homozygous state with cerebral malaria but not severe malarial anemia [10]. The TNF−238 and the TNF−308 A alleles were not linked in this population, and the relationship between the TNF−238 A allele and severe malarial anemia was unaffected by stratification for the TNF−308 genotype. On analysis of associations between the TNF−238 genotype
pathogenic process of severe malarial anemia is complex and incompletely understood. In many cases, the clinical features are consistent with prolonged infection due to poorly developed antimalarial immunity. Our hypothesis is that chronic low-grade production of TNF, in response to \textit{P. falciparum} parasitemia, induces dyserythropoiesis and contributes to the pathogenesis of malarial anemia. We have found plasma TNF levels not to be elevated in children presenting with severe malarial anemia compared with those with uncomplicated malarial fever. However, because of the relative chronicity of the illness, as compared with the typically acute presentation of cerebral malaria, and the short circulating half-life of TNF, standard plasma TNF measurements may be expected to shed little light on the question of whether severe anemia might result from excessive TNF production. Because it is extremely difficult to quantitate the production rates of TNF by plasma measurements and because it is not feasible to obtain bone marrow for histologic examination for the majority of patients, we opted to address this problem by examining for associations of severe malarial anemia with polymorphic markers in the TNF promoter region.

We found the TNF-238 A allele to be associated with susceptibility to severe malarial anemia, but not to cerebral malaria. In contrast, in the same population, we have previously found the neighboring TNF-308 A allele to be associated with cerebral malaria but not with severe malarial anemia [11]. Taken together, these findings suggest that the clinical outcome of malaria is influenced by complex genetic determinants near the TNF gene. Linkage disequilibrium of the TNF-238 A allele with several HLA alleles was identified, but none of these account for the association with susceptibility to severe malarial anemia. Of particular note, we found the TNF-238 A allele and HLA-B53 alleles to be linked, but their effects on susceptibility to severe malarial anemia work in opposite directions. This raises the intriguing possibility that in this case, natural selection has favored a haplotype that balances the risks of one immunologic characteristic against the benefits of another.

The location of the TNF-238 A allele in the TNF promoter region raises the possibility that it could influence constitutive TNF production directly [12]. However, this region in the mouse TNF promoter appears not to affect transcription [13], and a study of the TNF-238 A allele in human cells failed to reveal an effect on TNF response to short-term stimulation with bacterial lipopolysaccharide [14]. Thus, as with the TNF-308 A allele, where there is no proven function, it may be that the TNF-238 A allele serves as a marker for a functional polymorphism elsewhere in the TNF gene or in another nearby immunologically relevant gene. As TNF transcriptional regulation is known to be cell type- and stimulus-specific [15], further studies are required to determine the true functional importance of the allele in the context of chronic stimulation by \textit{P. falciparum}.

These findings in malaria relate to a general problem in the pathogenesis of infectious disease. There is clear evidence that TNF mediates acute symptoms such as fever and septic shock.
From experimental evidence, it appears probable that TNF also contributes to the cachexia and anemia of protracted infection, and this finding of an association between a polymorphic marker in the TNF promoter region and malarial anemia lends weight to this hypothesis. Further association studies in genetically distinct populations and studies in family groups would help to define precisely the genetic elements involved.

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References