CONCISE COMMUNICATION

Plasma Interleukin-10: Tumor Necrosis Factor (TNF)–α Ratio Is Associated with TNF Promoter Variants and Predicts Malarial Complications

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In individuals with severe malarial anemia, plasma levels of tumor necrosis factor (TNF)–α tend to exceed those of interleukin (IL)–10. In this study, IL-10:TNF plasma level ratios <1 were found to be a risk factor for both cerebral malaria and severe anemia (P = .009), whereas higher IL-10:TNF ratios were observed more frequently in hyperparasitic individuals. When considering allelic variants of the TNF promoter in children with severe malaria, carriers of the wild type more frequently had an IL-10:TNF ratio >1 (P = .008). In contrast, individuals with a mutation at position –238 of the TNF promoter (TNF-238A and TNF-176A/) consistently had lower IL-10 than TNF plasma levels (IL-10:TNF ratio <1; P = .001). Our results show that, in children with severe malaria, TNF promoter variants influence the balance of IL-10:TNF in the plasma, which, in turn, affects the outcome in terms of clinical complications.

The important role of the inflammatory cytokine tumor necrosis factor (TNF)–α in the pathogenesis of parasitic diseases has been demonstrated in several studies in vivo and in vitro [1]. In Plasmodium falciparum infection, appropriate levels of TNF exhibit antiparasitic effects [2], and a high TNF production capacity protects from severe malaria [3]. Excessive TNF levels are associated with complications such as cerebral malaria or severe anemia [4, 5]. It recently has been shown that a low ratio of plasma levels of the Th2 cytokine interleukin (IL)–10 and the Th1 cytokine TNF is associated with severe malarial anemia [6, 7].

Within the coding and the promoter region of the TNF gene, several single-nucleotide polymorphisms have been identified, and specific mutations in the promoter modify the transcriptional activity [8–10]. However, functional diversity resulting from these mutations has not been described consistently in all studies [11, 12]. It is unclear whether single-nucleotide polymorphisms in the TNF promoter region also influence IL-10:TNF plasma levels.

Single-nucleotide polymorphisms of the TNF promoter have been reported to be associated with susceptibility to or protection from severe malaria. Four mutations are transitions of adenine to guanine and are located at nucleotide positions –238, –244, –308, and –376, relative to the transcriptional start site. In a cross-sectional study, the mutation at position –308 (TNF-308A) was found to increase the risk of cerebral malaria [13]. Recent work by the same group has shown that, in 2 different African populations in Kenya and The Gambia, TNF-308A is an independent determinant of cerebral malaria [10]. In contrast, TNF-238A was associated with decreased susceptibility for this complication.

We analyzed whether the individual IL-10:TNF plasma level ratios might be predictive of severe malaria in carriers of different TNF variants. Furthermore, we investigated the influence of TNF promoter variants on the balance of IL-10 and TNF plasma levels.

Patients and Methods

Study group. One hundred children <11 years old with severe malaria and 100 control patients with mild malaria were enrolled in a case-control study. Thirteen individuals with mild malaria and 13 with severe malaria were excluded from further analysis because data regarding IL-10 or TNF plasma levels were missing. The study was conducted from 1995 to 1996 at the Albert Schweitzer Hospital in Lambarende, Gabon. Transmission of malaria parasites in this area is intense and stable. Patient groups and their clinical and...
parasitological indexes have been described in detail elsewhere [14]. Ethical clearance was obtained by the International Foundation of the Albert Schweitzer Hospital. All children were treated with antimalarial drugs after standard therapy schemes.

Severe malaria was defined as either severe malarial anemia (hemoglobin <50 g/L) or hyperparasitemia (>250,000 parasites/μL), or other signs of severe malaria, according to the definitions of the World Health Organization. Mild malaria was defined as reported [14].

**Genotyping of the TNF promoter region.** Genomic DNA was isolated from whole blood (EASY-DNA kit; Invitrogen, St. Louis). The proximal region of the TNF promoter (−69 to −376) was amplified by polymerase chain reaction (PCR) with the primers TNF-α.F (5'-TTCTGCATCTGTCTGGAA) and TNF-α.R (5'-CAGCGGAAAACTTTCTTGGT) for 4 min at 95°C, then we ran 35 cycles of 40 s at 95°C, 40 s at 56°C, 1 min at 72°C, and 6 min at 72°C. Amplicons were monitored on agarose gels and were cross-linked onto nylon membranes by exposure to ultraviolet light. The membranes were hybridized with sequence-specific oligonucleotides (SSOPs) labeled with digoxigenin-11-2-tetramethylammoniumchloride at temperatures specific for each SSOP, and signals were detected with an anti-digoxigenin alkaline phosphatase conjugate (Fab fragments). The reliability of SSOP typing was controlled by DNA sequencing of a randomly selected panel of samples. PCR products with ambiguous hybridization patterns underwent DNA sequencing as well, for definitive assignment of alleles.

**Cytokine assays.** Blood concentrations of TNF and human IL-10 were measured with commercially available ELISA kits (Flexia; BioSource, Ratingen, Germany). Detection limits were defined as 1 pg/mL for both cytokines, and values below this level were assigned a value of 0. For measurement of the TNF production capacity, whole-blood samples (700 μL) were cultured (20 h, 37°C, and 5% CO₂) with or without stimulation (10 μg/mL phytohemagglutinin [PHA]; Sigma, Deisenhofen, Germany). TNF production capacity was calculated by subtracting the value measured in the unstimulated sample from that measured in the PHA-stimulated sample.

**Statistical analysis.** Statistical analyses were performed by the StatView 5.0 and JMP 3.0 software (SAS Institute, Cary, NC). χ² tests were calculated to estimate differences of qualitative variables. The Mann-Whitney U test was applied as nonparametric test, to compare TNF and IL-10 plasma levels in individuals with different TNF promoter variants. A 2-tailed P value <.05 was considered significant.

For the identification of effectors of IL-10:TNF plasma level ratios, a multiple regression analysis was performed. The model was achieved by forward stepwise inclusion of the bivariate variables hyperparasitemia, anemia, cerebral malaria, and hypoglycemia (probability to enter .25 and probability to leave .1).

**Results**

**Distribution of TNF promoter variants in individuals.** The TNF promoter region was typed for the G→A transitions occurring at positions −238, −244, −308, and −376. DNA sequencing of 10 randomly selected samples proved the reliability of the SSOP typing. The allelic frequencies met the expectations of the Hardy-Weinberg law. All TNF variants occurred in polymorphic frequencies (wild type, .822; −238A, .023; −244A, .034; −308A, .057; and −238A/−376A, .057). The wild type (indicated with a superscript "wt") occurred in virtually all individuals (97%) and in 68% of them homozygously. Other variants were never found as homozygous genotypes. The mutation −376A was in complete linkage with −238A (TNF−238A/−376A), as confirmed by cloning and sequencing of all samples with both the −238A and the −376A mutations in which genotypes cannot be determined unambiguously by SSOP typing.

The frequencies of carriers of distinct TNF variants did not differ significantly in the groups with mild or severe malaria (mild vs. severe malaria: TNFwt, 96.6% vs. 97.7%; TNF−238A, 2.3% vs. 6.9%; TNF−244A, 8.1% vs. 5.8%; TNF−308A, 14.9% vs. 8.1%; and TNF−238A/−376A, 9.2% vs. 16.1%).

**TNF plasma levels, TNF production capacities, and TNF promoter variants.** In 87 children with severe malaria, TNF plasma levels and TNF production capacities were measured at admission and correlated to the TNF promoter genotypes. TNF plasma levels were not significantly associated with any of the TNF variants (median plasma levels [interquartile range]: TNF−238A, 31.2 [207–1409] pg/mL; TNF−244A, 476 [217–632] pg/mL; TNF−308A, 354 [203–730] pg/mL; and TNF−238A/−376A, 371 [207–1207] pg/mL). Similarly, TNF production capacity and IL-10 plasma levels at admission were comparably high in children with different TNF promoter alleles (data not shown).

Six months after initial enrollment of patients into the study, measurement of TNF levels was repeated in 64 children during an infection-free interval. Again, TNF plasma levels were not dependent on distinct TNF promoter variants (homozygous TNFwt, 80 [56–156] pg/mL; TNF−238A, 89 [65–401] pg/mL; TNF−244A, 119 [82–156] pg/mL; TNF−308A, 80 [60–130] pg/mL; and TNF−238A/−376A, 62 [32–124] pg/mL).

**Relation of the IL-10:TNF plasma level ratio and TNF promoter mutations.** The IL-10:TNF plasma level ratio has been shown to be indicative of malarial complications [6, 7]. Therefore, we looked at possible associations of the IL-10:TNF ratio with TNF promoter variants. At admission, 63 children with severe malaria had lower IL-10 levels than TNF plasma levels (IL-10:TNF <1), and 24 children had higher IL-10 than TNF plasma levels (IL-10:TNF >1; table 1).

The distribution of TNF promoter variants in individuals with IL-10:TNF ratios <1 differed markedly from that in individuals with an IL-10:TNF ratio >1. A higher proportion of individuals homozygous for TNFwt had IL-10:TNF ratios >1, compared with those with other TNF promoter variants.
Table 1. Relationship between interleukin (IL)-10 and tumor necrosis factor (TNF)-α plasma level ratios and TNF promoter alleles in patients with severe malaria.

<table>
<thead>
<tr>
<th>TNF promoter variants</th>
<th>IL-10:TNF ratio, median (interquartile range)</th>
<th>IL-10:TNF &lt;1 (n = 63)a,b</th>
<th>IL-10:TNF &gt;1 (n = 24)c</th>
<th>Pd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type homozygous</td>
<td>0.6 (0.2–1.5)</td>
<td>36 (57.1)</td>
<td>21 (87.5)</td>
<td>.008</td>
</tr>
<tr>
<td>TNF&lt;sup&gt;−238A/−276A&lt;/sup&gt;</td>
<td>0.4 (0.1–0.6)</td>
<td>14 (22.2)</td>
<td>0 (0.0)</td>
<td>.012</td>
</tr>
<tr>
<td>TNF&lt;sup&gt;−376A&lt;/sup&gt;</td>
<td>0.9 (0.1–0.6)</td>
<td>5 (7.9)</td>
<td>2 (8.3)</td>
<td>NS</td>
</tr>
<tr>
<td>TNF&lt;sup&gt;−238A&lt;/sup&gt;</td>
<td>0.4 (0.2–1.2)</td>
<td>4 (6.4)</td>
<td>1 (4.2)</td>
<td>NS</td>
</tr>
<tr>
<td>TNF&lt;sup&gt;−276A&lt;/sup&gt;</td>
<td>0.7 (0.3–0.9)</td>
<td>6 (9.5)</td>
<td>0 (0.0)</td>
<td>NS</td>
</tr>
</tbody>
</table>

NOTE. NS, not significant.

a Data are no. (%) of patients.

b Two individuals were carriers of 2 TNF promoter variants other than wild-type TNF.

c P values calculated by χ² tests of variants, compared with all other variants.

d After correction for 5 comparisons.

(P = .008). Children carrying the −238A mutation had a median IL-10:TNF ratio of 0.5 (interquartile range, 0.2–0.6), which was always <1 (−238G vs. −238A; P = .002). This was independent of the occurrence of a second mutation at position −376 (occurrence of −376A, TNF<sup>−238A/−276A</sup> vs. TNF<sup>−238A</sup>, P = .012; absence of −376A, TNF<sup>−238A</sup> vs. TNF<sup>−276A</sup>, not significant).

Because of the scattered distribution of both IL-10:TNF ratio values and TNF variants, medians of ratios were not significantly different from each other (Mann-Whitney U test).

IL-10: TNF plasma level ratio and complications in severe malaria. Complications of severe malaria included hyperparasitemia, anemia, cerebral malaria, and hypoglycemia. Hyperparasitemia was the most frequent condition of severe malaria, and 32% of hyperparasitemic children had a higher IL-10 than TNF plasma level (figure 1). In these individuals, IL-10:TNF plasma level ratios were significantly higher than in children without hyperparasitemia (Mann-Whitney U test, P = .003). In contrast, children with severe anemia had lower IL-10:TNF ratios than those without anemia (Mann-Whitney U test, P = .009). Children with cerebral malaria consistently had IL-10:TNF ratios <1. Similarly, in individuals with hypoglycemia (blood glucose <40 mg/dL), the TNF level was, with the exception of 1 patient, always higher than that of IL-10.

To identify the relevant factors independently associated with the IL-10:TNF plasma level ratios, a multiple regression model with the 4 effects of hyperparasitemia, anemia, cerebral malaria, and hypoglycemia, applied individually and combined with each other, was performed. A low IL-10:TNF plasma level ratio was identified as a predictor of anemia (F ratio, 4.5; P < .04), and high ratios were predictive of hyperparasitemia (F ratio, 4.0; P < .05).

Discussion

Our findings show that a mutation at position −238 of the TNF promoter strongly correlates with a lower IL-10:TNF plasma level ratio in individuals with mutually exclusive different manifestations of severe malaria or exclusive combinations of malarial complications. The multiple regression model by forward stepwise inclusion of the manifestations (probability to enter, .25; probability to leave, .1) identified hyperparasitemia to correlate positively and anemia to correlate negatively with IL-10:TNF plasma level ratios. A, severe anemia (hemoglobin blood concentration <50 g/L); C, cerebral malaria; G, hypoglycemia (blood glucose level <40 mg/dL); P, hyperparasitemia (>250,000 parasites/µL).
plasma level ratio, which mediates susceptibility to severe complications of malaria. The group of children with complications included those with an exclusive mutation at position −238 (TNF−238A) and those with the additional mutation at position −376 (TNF−238A−376A). Because of the tight linkage of −238A and −376A, it is impossible to estimate the independent influences of the 2 mutations.

In this study, children with mild and those with severe malaria had similar distributions of TNF promoter single-nucleotide polymorphisms with susceptibility for or protection from severe malaria have been reported so far. In a case-control study in The Gambia, children homozygous for the TNF−308A allele had an increased risk of lethality due to cerebral malaria [13]. In the same study group, TNF−238A−376A was identified to be a risk factor of cerebral malaria. This observation has recently been confirmed in a Kenyan population [10]. On the other hand, TNF−238A was associated with protection from cerebral malaria in the Kenyan but not in the Gambian population [10]; in the latter, it is a marker of severe malarial anemia [15]. In Sri Lanka, the TNF−308A variant was found to be associated with severe disease, either of malarial or other infectious origin, in comparison to a healthy condition or to uncomplicated malaria [16].

The anti-inflammatory cytokine IL-10 is secreted simultaneously or shortly after the proinflammatory cytokine TNF, and IL-10 inhibits the release of TNF. In this study, all children with severe malaria carrying a mutation at position −238 had lower IL-10 than TNF plasma levels. In contrast, children homozygous for TNF−308A more frequently had higher IL-10 than TNF plasma levels. Interestingly, neither the TNF nor the IL-10 plasma level was exclusively associated with the occurrence of 1 of the TNF promoter variants, whereas the IL-10:TNF balance was altered. TNF and IL-10 are both secreted by monocytes/macrophages, B cells and T cells, and it is conceivable that slight up-regulation of TNF and relative down-regulation of IL-10 secretion in severe malaria is only detectable as a switch of the TNF:IL-10 level ratio at an individual level.

TNF secretion in vitro was previously found to depend on particular TNF variants. In a human B cell line, TNF−308A was a stronger transcriptional activator than TNF−308 [8]. The mutation at position −376A has been identified as a binding site of the helix-turn-helix transcription factor OCT-1, which alters basal gene expression in transfected human monocytes [10]. So far, it has not been confirmed in vivo that TNF expression per se is controlled by its promoter variants. Our results show now that the TNF variants are determinants of the IL-10:TNF plasma level balance. On the other hand, a low IL-10:TNF plasma level ratio appears to be a risk factor for anemia in children with severe malaria [6, 7]. This is in agreement with our results, which show an almost exclusive association between an IL-10:TNF ratio <1 and severe malarial anemia, but also with cerebral malaria and hypoglycemia.

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References