Association of a Single Nucleotide Polymorphism in the C-Reactive Protein Gene (–286) with Susceptibility to Plasmodium falciparum Malaria

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The role of inflammation in malaria pathogenesis is not fully understood, although C-reactive protein (CRP) may have a negative influence on host immunity to infections. An upstream polymorphism, –286 (C > T > A), in the CRP gene is known to influence CRP levels. In this study, a cohort of 192 Sudanese donors, followed for malaria infection for 9 years, had their CRP –286 gene locus genotyped by pyrosequencing. The number of malaria episodes experienced by each individual over the study period was used as an index for malaria susceptibility. The prevalence of the CRP alleles A, C and T were 21%, 52% and 27%, respectively. Importantly, the A-allele, unlike the C- and T-alleles or CRP genotypes, was significantly associated with an increased number of malaria episodes, P = 0.007. The proportion of A-allele carriers among donors not known to have had malaria during the study period was 18%, whereas it was 43% and 63% among donors who had experienced 1–4 and ≥5 malaria episodes, respectively, over the same period (P = 0.002). Furthermore, the A-allele was associated with higher parasite counts. In conclusion, the CRP –286 A-allele was associated with an increased susceptibility to uncomplicated Plasmodium falciparum malaria.

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INTRODUCTION:
Malaria imposes an unlimited burden in communities, notably in sub-Saharan Africa and other equatorial regions (1). Over time, malaria infections have left imprints in the human genome that evolved into single nucleotide polymorphisms (SNPs) of genes involved in protection/susceptibility to malaria (2). The role of inflammation in malaria pathogenesis is not well understood (3).

The role of inflammation in malaria pathogenesis is not fully understood, although C-reactive protein (CRP), a nonspecific acute-phase protein, is a classic marker for inflammation. CRP is found in plasma in trace amounts in healthy individuals, and increases in concentration 100- to 10,000-fold during acute inflammation and in disease situations including infections (4,5). CRP is produced predominantly by the liver and its production is induced by the proinflammatory cytokine interleukin 6 (IL-6) (6,7).

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Recent studies suggest that CRP acts through FcR to reduce inflammation and protect against certain autoimmune diseases (8). Structurally, the CRP has a sequence homology (shared domains) to the FcγR binding regions of IgG (9). Furthermore, CRP has high affinity to the R131-allele product of the FcγRIIA gene (10), an allele that has been associated with protection against malaria (11). Thus, CRP could be a natural competitor for antimalarial IgG binding to phagocytic and other FcγR-containing cells. Interestingly, both CRP and FcγR genes are located on chromosome 1.

Although the pathological role of CRP in cardiovascular diseases has been thoroughly investigated (12), its role in parasitic infections, more specifically in ma-
laria, has been less investigated (13). The involvement of CRP in human defense against infection is partially due to its capacity to bind to phosphorylcholine in membranes of microorganisms (14,15). In malaria, the secretion of CRP is induced by proinflammatory cytokines secreted by host monocytes/macrophages (16), and strong correlations have been found between CRP levels and parasitemias (17). Furthermore, apparently healthy African children were found to have higher levels of CRP compared with children in Europe (18) and Papua New Guinea (13), which might indicate chronic malarial parasitization of the former population.

The CRP gene is highly polymorphic, with SNPs identified at several loci; however, the triallelic –286 (C > T > A) SNP (rs3091244) is strongly associated with the plasma CRP concentration (19). The A-allele was found to be associated with higher basal levels of CRP (20). The distribution of CRP genotypes and alleles are geographically and ethnically different. Previous studies showed that the A-allele is more common in African than in white populations (21), and would thus be expected to confer a survival advantage for the inhabitants of malaria endemic areas. We examined three SNPs; –286 C > T > A, –717 T > C and +1444C > T in material obtained from Daraweesh village in eastern Sudan. The –286 SNP was found to be different in ethnic groups with varying susceptibility to malaria (22); thus, it was selected for further analysis in this study.

MATERIALS AND METHODS

Study Area and Population

This study was carried out in Daraweesh village (the total population was >550 individuals in 2005), eastern Sudan, between 1991 and 2005. The inhabitants are descendant of the Fulani tribe from Higher Volta (Burkina Faso) in West Africa. The ethnicity in Daraweesh has been maintained by a high rate of intermarriage. In Daraweesh, malaria transmission is seasonal, starts in September, peaks in October or November and declines in January to a baseline of few sporadic cases, a pattern that continues to August. The malaria incidence is markedly unstable; severe epidemics occur in flood years, whereas in drought years malaria cases are almost absent. In Daraweesh more than 98% of the malaria infections were due to *Plasmodium falciparum*. An individual in the village is unlikely to experience more than one malaria episode in the same season (23,24).

Study Design

The Daraweesh project was a total population longitudinal study begun in 1991, aiming to document seasonal incidence of malaria in the village, with a cohort-based component for study of the natural immune response to malaria. The study design has been thoroughly described (23,25). Malaria infection was documented by active and passive surveillances during the malaria season. Data used in this study were obtained over a 9-year period between 1991 and 2005. This study used longitudinally collected data for 3 parameters: the number of malaria episodes, the parasite density and the temperature. For consistency in execution and method, we used the parasite density data obtained in the period between 1993–1996, when the density was scored semiquantitatively (+, + + , + + + and + + + + + ) and in 1998–1999, when parasite count was used. However, during both periods heavy malaria transmission occurred in the area. The plasma samples used in this study were obtained in May 2005 from 192 donors. All donors had no malaria infection at the time of sampling, demonstrated by polymerase chain reaction (PCR) analysis of the samples. The number, age and sex of the patients are shown in Table 1.

Longitudinal Clinical and Parasitological Data

Clinical examination and malaria diagnosis. Malaria was defined as fever with measurable temperature of ≥37.5°C or a recent history of fever and microscopically detectable asexual *P. falciparum* parasites of any count. Individuals complaining of fever or symptoms suggestive for malaria were clinically examined, then oral temperature (in young children axillary temperature) and body weight were measured; thereafter, a blood smear was taken. Thin and thick blood films were stained with Giemsa and examined microscopically for detection of both sexual and asexual stages of *P. falciparum* parasites, using the WHO standard procedure (26). Slides were read and revised by expert microscopists using light microscopy. Malaria patients were mostly treated with chloroquine alone (10-10-5 mg/kg/day, over 3 d); however, in the last two malaria seasons,
DNA Preparation

DNA was extracted from blood with Chelex-100. Briefly, around 25 μL of peripheral blood was incubated at 4°C in 1 mL of 0.5% Saponin in phosphate-buffered saline overnight, and then washed in 1 mL phosphate-buffered saline at 4°C for 15–30 min. The pellets were boiled in 200 μL of 5% Chelex-100 in water for 15 min, and after centrifugation at 8,000g for 3 min the DNA was collected in supernatants and stored at –20°C.

CRP Genotyping with Pyrosequencing

The –286 C > T > A CRP polymorphism (rs3091244) was analyzed by the pyrosequencing method (22). PCR primers were designed to amplify the –286 C > T > A polymorphism, using the forward and reverse primer (MWG-Biotech AG, Ebersberg, Germany). The PCR amplification was performed in a 40-μL reaction using 8 μL of genomic DNA, 20 μL iProof™ High-Fidelity Master Mix (BIO-RAD Laboratories, Hercules, CA, USA), and 1.5 μL of each primer (10 pmol/μL). The PCR was carried out in an Eppendorf Mastercycler® (Eppendorf AG, Hamburg, Germany) using a 30-s denaturation at 98°C followed by 35 cycles with 98°C for 10 s, 59°C for 20 s and 72°C for 15 s. The final extension was at 72°C for 5 min. The sequencing primer was 5′-GTG CAC CCA GAT GCC-3′ (MWG-Biotech AG), and the nucleotide dispensation order was GCACGTACAGT. Sequencing was carried out as instructed by the protocol using the PSQ™96MA pyrosequencing apparatus.

Statistical Analysis

Sigma Stat software was used for data analysis. Kruskal–Wallis one-way analysis of variance on ranks was used for comparison of age, parasite count and number of malaria episodes between the different CRP –286 allele/genotype carriers. Proportions and rates were tested by Chi-square test or Fisher exact test and correlations were performed by using Pearson product moment correlation.

RESULTS

Longitudinal Clinical Data

The age of the donors ranged between 6 and 78 years, with a median (25–75%) age of 19 (12–34.5 years) at the time of blood sampling in May 2005. There were no significant age differences between the carriers of the different CRP –286 genotypes (P = 0.382, Kruskal–Wallis one-way analysis of variance on ranks). Although females predominated in this study (male/female ratio 67:125), the sex ratios were comparable between the groups. The number of malaria episodes experienced by each individual over the study duration was not correlated with the donor age at the time of sampling (r = –0.103, P = 0.154). However, when the analysis was limited to individuals born at least 1 year before the beginning of the study in 1991, there was an inverse relationship between age (year of birth) and number of malaria episodes (r = –0.266, P = 0.003, n = 121), that is, older individuals had a lower number of malaria episodes.

Selection of SNP for Analysis in the CRP Gene

There are several SNPs in the CRP gene that could be investigated. However, in a previous study, at least 30 SNPs were identified, 9 of which were found to correlate with plasma CRP levels, but there was strong linkage disequilibrium between these SNPs. With stepwise selection, the triallelic (C > T > A) SNP –286 (rs3091244) remained strongly associated with plasma CRP concentration, independent of other polymorphisms (19). In our previous study, we examined the –286 SNP in addition to another two polymorphisms, –717 T > C (rs2794521) and +1444C > T (rs1130864). Because the prevalence of –286 SNP, unlike that of the latter two polymorphisms, was significantly different in sympatric ethnic groups with varying susceptibility to malaria in Sudan (22), this SNP was selected for further analysis.

The CRP –286 Genotypes and Frequency of Malaria Episodes

The frequencies of the six CRP genotypes (AA, AC, AT, CT, CC and TT) in Daraweesh are shown in Table 1. The lowest and highest frequencies were AA, 0.052 (n = 10) and CC, 0.302 (n = 58), respectively. The number of malaria episodes (median, 25–75%) experienced by the carriers of the above genotypes were comparable, P = 0.076, (Table 1).
**CRP –286 C- > T- > A-Alleles and Frequency of Malaria Episodes**

The frequencies of CRP –286 alleles in decreasing order were; C-allele (52.08%), T-allele (26.8%) and A-allele (21.1%). During the 9 years of study, comparison of the number of malaria episodes (median, 25–75%) between A-allele carriers, (n = 71) 2, 1–3, and non–A-allele carriers (n = 121) 1, 0–2, showed that the difference was strongly statistically significant (P = 0.007) (Figure 1). The number of malaria episodes was comparable between the C-allele (n = 50) 2, 1–3 and non–C-allele carriers (n = 142) 1, 0–2, (P = 0.205). Similarly, between the T-allele (n = 87) 2, 0–3.0 and non–T-allele carriers (n = 105) 1, 0–2, P = 0.688. Although only 18.2% (10 of 55) of the patients who did not develop malaria during the follow-up were A-allele carriers, 63% (5 of 8) of the donors who had experienced the highest number of malaria episodes (≥5 episodes) were A-allele carriers. Approximately 43% (56 of 129) of the donors who had experienced between 1 to 4 malaria episodes were A-allele carriers (Figure 2). The overall differences in the proportions between the A- and non–A-allele carriers in all ranks of infections (0 to ≥5 episodes) were significant, P < 0.002.

**CRP Genotypes/Alleles, Parasite Density and Oral Temperature**

The parasite count (malaria seasons 1998–1999 [n = 140]) did not differ between the carriers of the different CRP genotypes (P = 0.384), but there were statistically significant higher parasite counts among A-allele carriers (n = 65) (2460.0, 455.5–9680.8 parasites/μL of blood [median, 25–75%]) compared with the non–A-allele carriers (n = 75) (983.0, 244.8–4950.0), P = 0.039. The parasite counts were comparable between the C-allele carriers and non–C-allele carriers (P = 0.898) and between the T-allele carriers and non–T-allele carriers (P = 0.279) (Figure 3). However, using the semiquantitative parasite density scoring (malaria seasons 1993–1996), we found no significant differences between the CRP phenotype between the different CRP –286 phenotypes; allele carriers (data not shown).

There was no association between the parasite count and oral temperature (r = –0.0008, P = 0.992 [n = 171]), although there was a positive correlation between the oral temperature and the semiquantitative scoring of parasite density (r = 0.279, P < 0.001 [n = 360]).

**Number of Malaria Episodes and Distribution of CRP Genotypes/Alleles between Carriers and Noncarriers of Sickle Hemoglobin**

The sickle hemoglobin (Hb AS) carriers (n = 33) experienced a similar number of malaria episodes (median, 25–75% [2.0, 0.0–2.25]) as the Hb AA carriers (1.0, 0.0–2.75 [n = 159]), P = 0.944, even when the analysis was limited to individuals born before the year 1991 (P = 0.834). Although only one of the AA genotype carriers (10%, 1 of 10) was a carrier of Hb AS, the proportions of the latter among the other CRP genotype carriers were not significantly different (range 12.5–20.8%), P = 0.964. The frequency of the Hb AS trait was comparable between the CRP –286 A-allele (16.9%, 12/59) and non–A-allele (17.3%, 21/100) carriers (P = 0.906); similarly, Hb AS was not associated with C- or T-alleles (data not shown).

**DISCUSSION**

The overwhelming effect of malaria on man over history has imposed evolutionary alterations of the human genome (27), particularly of genes involved in defense mechanisms, to decrease the disease burden. In this study, we found that the CRP –286 A-allele was associated with remarkably increased susceptibility of the Sudanese donors to malaria. This could be one reason for the low prevalence of the A-allele in this area. The CRP –286 A-allele influence on malaria susceptibility in this cohort was stronger than the effect of Hb AS, the latter known to be associated with reduced malaria burden. Furthermore, although A-allele carriers account for only 18% of the malaria-resistant individuals (not
known to have had malaria in 9 years), they accounted for 63% of the most susceptible individuals in the village. This might be the first report that strongly indicates a role for a CRP polymorphism and possibly inflammation in malaria susceptibility. However, the effect of the CRP on susceptibility to malaria might be equally due to modulation of immunity, independently of inflammation.

The dual role of host immunity in protection from as well as in pathogenesis of malaria is known (28). In contrast, the role of inflammation in malaria protection and pathogenesis is not well defined. The implication of plasma CRP levels and inflammation in pregnancy-associated anemia in the tropics has been reported (29), otherwise the literature lacks data relating inflammation or CRP with malaria pathogenesis. Unlike acquired immunity, inflammation is a nonspecific response to different diseases and there are no specific units for measurement of inflammation. However, CRP is one of the best markers for inflammation (6). Polymorphisms in the promoter region of the CRP gene (–286 C > T > A) affect the plasma CRP level, and the A-allele has been found to be associated with high CRP concentrations (19, 20).

In this study, the A-allele compared with the non–A-allele carriers had the tendency to have higher parasite density when they developed a new malaria attack or upon recrudescence of parasitemia. The association of CRP level with malaria infection has been previously reported (17,18,30). However, the unique observation in this study is that the association of the A-allele with parasitemia was based on several infections in the same individual and included the parasite density following treatment failure. There was no association, however, between the CRP genotypes/alleles or the parasite density and body temperature. This result is in line with our observation that high parasitemia does not always parallel malaria symptoms, including fever.

Taken together, the above findings are suggestive of a strong association between CRP and susceptibility to malaria. Possible explanations for this association are (a) inflammation constitutes part of malaria pathogenesis and CRP plays a role in the inflammatory process; (b) CRP modulates host humoral immunity (it has been suggested that CRP binds FcγR in immune cells,
thereby interfering with the immune response to the malaria parasite by inhibiting the binding of malaria-specific IgG antibodies [9]) or (c) CRP modulates the host cellular immunity by altering the proinflammatory (IL-6)/antiinflammatory (IL-10) cytokine balance (16). These effects are not mutually exclusive, however, and are mediated by structurally normal CRP, that is, the SNP is in the noncoding promoter sequence. Thus, what matters is the plasma CRP level, regardless of the causative polymorphism. Additional evidence for the role of CRP in malaria susceptibility was our finding that the A-allele is less frequent in the Fulani than their neighbors in different endemic settings and other functional noncoding and structural coding CRP gene mutations.

In conclusion, in this longitudinal cohort-based study in Daraweesh, we found a strong association between increased malaria susceptibility and CRP –286 A-allele carriage. In addition, A-allele carriers had significantly higher parasitemia compared with non–A-allele carriers. Consequently, these data suggest a strong association between inflammation and malaria pathogenesis, an association that is greater than previously described.

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DISCLOSURE
The authors declare that they have no competing interests as defined by Molecular Medicine, or other interests that might be perceived to influence the results and discussion reported in this paper.

REFERENCES
nucleotide polymorphism in the promoter region
of the C-reactive protein gene associated with the
22. Israëllson E, et al. (2009) Marked differences in
CRP genotype frequencies between the Fulani
veillance in a Sudanese village highlights unex-
pected variation in individual disease susceptibil-
ity and outbreak severity. Parasitology. 129:263–71
24. A-Elbasit IE, Elbashir MI, Khalil IF, Alfrangis M,
Giha HA. (2006) The efficacy of sulfadoxine-
pyrimethamine alone and in combination with
chloroquine for malaria treatment in rural Eastern Sudan: the interrelation between resistance,
febrile malaria episodes in an area of unstable
Hyg. 94:645–51.
microscopy, part 1. Geneva: World Health Organ-
ization.
27. Fortin A, Stevenson MM, Gros P. (2002) Suscepti-
bility to malaria as a complex trait: big pressure
28. Artavanis-Tsakonas K, Tongren JE, Riley EM.
(2003) The war between the malaria parasite and
the immune system: immunity, immunoregula-
133:145–52.
29. van den Broek NR, Letsky EA. (2000) Etiology of
anemia in pregnancy in south Malawi. Am. J.
levels and falciparum malaria. Trans. R. Soc. Trop.
to Plasmodium falciparum malaria of Fulani of
Burkina Faso (west Africa) is associated with low
frequencies of classic malaria-resistance genes.
hemoglobin (HbS) allele and sickle cell disease: a HuGE review. Am. J. Epidemiol. 151:839–45.
33. Dolo A, et al. (2005) Difference in susceptibility to