Inverse Relationship of Plasma Prostaglandin E\textsubscript{2} and Blood Mononuclear Cell Cyclooxygenase-2 with Disease Severity in Children with \textit{Plasmodium falciparum} Malaria

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The Journal of Infectious Diseases 2001; 183:113±8

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0022-1899/2001/18301-0014$02.00

Prostaglandins (PGs) derived from inducible cyclooxygenase (COX)-2 are important proinflammatory mediators of the host-immune response to infection. Since the role of host-derived PG in human malaria is unknown, plasma bicyclo-PGE\textsubscript{2}, (a stable catabolite of PGE\textsubscript{2}), peripheral blood mononuclear cell COX-2 protein, and mRNA were measured in Gabonese children with and without malaria ($n = 129$). Relative to healthy children, bicyclo-PGE\textsubscript{2} and COX-2 protein were lower in children with mild ($P = .007$ and $P = .026$, respectively) and severe malaria ($P = .002$ and $P = .010$, respectively). COX-2 mRNA was also reduced in children with malaria. Investigation of COX-2 regulatory cytokines revealed an inverse correlation ($P < .001$) between plasma levels of bicyclo-PGE\textsubscript{2} and interleukin (IL)-10, a cytokine that suppresses COX-2 expression. On the basis of these results, elevated PGE\textsubscript{2} in healthy malaria-exposed children may protect against malaria, whereas IL-10-induced decreases in PGE\textsubscript{2} during acute malaria may increase susceptibility to severe disease.

Malaria is estimated to cause 300–500 million new clinical cases annually and to result in 1.5–2.7 million deaths \cite{1}. Most of the morbidity and mortality associated with malaria occurs in children <5 years old and relates to their nonimmune status. Severe falciparum malaria in African children is frequently characterized by hyperparasitemia, severe anemia, hypoglycemia, cerebral malaria, and respiratory distress \cite{2, 3}. In areas where \textit{Plasmodium falciparum} transmission is hyperendemic, such as Lambare\textsuperscript{n}e, Gabon, the clinical manifestations of severe childhood malaria are typically severe anemia and hyperparasitemia \cite{4}. The underlying pathophysiological determinants of severe malaria are largely unknown.

Although not well characterized as mediators of the host immune response in malaria, increased prostaglandin (PG) formation in malaria has been inferred because of the near universal presence of fever. PGs are important modulators of the inflammatory response in a number of diseases, including asthma, arthritis, cardiovascular disease, hypotension and shock, leishmaniasis, neurological disorders, and cancer \cite{5}. Release of PG into the localized cellular milieu is also important for the regulation of vascular function, fever, inflammation, and modulation of immune reactions \cite{5}.

After cellular activation, arachidonic acid (AA) is liberated from membrane glycerophospholipids by the actions of phospholipase A\textsubscript{2} \cite{6}. Conversion of AA to PG (e.g. PGE\textsubscript{2}, PGF\textsubscript{2\alpha}, PGE\textsubscript{1}, PGD\textsubscript{2}, prostacyclin, and thromboxane-A\textsubscript{2}) is catalyzed by 2 isoforms of cyclooxygenase (COX-1 and COX-2; also termed PGH\textsubscript{2} synthase–1 and –2) \cite{5}. COX-1 and COX-2 (M, 70–72 kDa) have ~60% homology at the amino acid level and are encoded by separate genes \cite{5, 7}. In general, COX-1-generated PGs are important in physiological homeostasis, whereas COX-2-generated PGs are important in inflammation and host defense \cite{8}.

Many of the mechanistic properties associated with PG have been studied by evaluating the effects of PG synthesis inhibition by nonsteroidal anti-inflammatory drugs (NSAIDs). Although NSAIDs are generally administered for the discomfort associated with pain and fever, attenuation of these signs and symptoms can be associated with adverse effects. For example, NSAID use is associated with the progression of invasive group A streptococcal infections to multiorgan failure and shock (reviewed in \cite{9}). Moreover, pretreatment of humans with NSAIDs before injection with endotoxin results in 4–10-fold higher plasma tumor necrosis factor (TNF)–α levels than in control subjects given endotoxin alone \cite{10}. Recent studies in a murine model of cerebral malaria illustrate that blockade of PG formation with aspirin is associated with adverse effects \cite{11}. Thus,
inhibition of PG synthesis in the context of host defense may have adverse effects.

Symptomatic treatment of children with malaria generally includes use of antipyretics, such as acetaminophen (paracetamol). The antipyretic effects of acetaminophen appear related to the inhibition of PGE₂ production within the brain, while having little or no effect on peripheral PG production [12]. However, in a recent study in Gabonese children with malaria, treatment with acetaminophen provided no significant reduction in fever, compared with only manual fanning of the children [13]. Treatment with acetaminophen was associated with prolonged parasite clearance time and decreased TNF and oxygen radical production [13]. These results are consistent with investigations showing that compounds that inhibit PG production can also suppress certain leukocyte functions, such as chemotaxis, phagocytosis, reactive oxygen species generation, and bacterial killing (reviewed in [9]). To characterize PGE₂ in malaria, we measured plasma PGE₂ levels in Gabonese children with varying degrees of malarial severity. We also examined COX-2 mRNA and protein in peripheral blood mononuclear cells (PBMC) and plasma levels of selected cytokines that could potentially regulate COX-2.

Subjects and Methods

**Study participants.** Children (n = 129; 2–7 years old) were recruited at the Albert Schweitzer Hospital in Lambaréné, an area where *P. falciparum* transmission is hyperendemic [4]. Healthy donors (n = 25: 12 boys and 13 girls) were from Lambaréné and the surrounding area. Thick blood films were used to verify that the children were free of malarial parasites. Patients who sought treatment for falciparum malaria were categorized as mild (n = 54: 29 boys and 25 girls) or severe (n = 50: 27 boys and 23 girls) malaria cases. Classification of severity was done according to modified World Health Organization (WHO) guidelines [14]. Inclusion criteria for severe cases were >250,000 parasites/μL, regardless of packed cell volume (PCV), or >100,000 parasites/μL in the presence of severe anemia (hemoglobin, ≤6.0 g/dL or PCV ≤20%). All blood samples were obtained before treatment with antimalarials. Although use of salicylates is rare in this community, a few cases were identified and were excluded from our analysis, since these treatments for falciparum malaria were categorized as mild (n = 54: 29 boys and 25 girls) or severe (n = 50: 27 boys and 23 girls) malaria cases. Classification of severity was done according to modified WHO criteria [14], subjects with a positive diagnosis for *P. falciparum* were categorized as mild (n = 29 children among groups were made by the Mann-Whitney U test with statistical significance set at *P* < .05. Linear regression was used to determine the association among variables with statistical significance set at *P* < .05.

Results

**Patient characteristics.** The study population (n = 129; 2–7 years old) was recruited at Albert Schweitzer Hospital in Lambaréné in the Central African rain forest where *P. falciparum* is hyperendemic [4]. Healthy children (n = 25) were recruited during routine checks from an ongoing longitudinal study in Lambaréné and were free of malarial parasites or other known illnesses for ≥2 months before study inclusion. Children who came to the Albert Schweitzer Hospital were evaluated for malaria by clinical and laboratory measures. On the basis of modified WHO criteria [14], subjects with a positive diagnosis for
falciparum malaria were grouped as having mild (n = 54) and severe malaria (n = 50). There were no cases of cerebral malaria. Table 1 summarizes the clinical and laboratory findings for the children with malaria.

**Plasma levels of bicyclo-PGE₂.** Systemic PGE₂ production (measured as plasma bicyclo-PGE₂) was analyzed in children with various degrees of malarial severity. Bicyclo-PGE₂ levels were inversely related to disease severity (figure 1). Relative to the healthy control group, plasma levels of bicyclo-PGE₂ were reduced in the mild malaria group (P = .007) and in the severe malaria group (P = .002; figure 1). In addition, plasma bicyclo-PGE₂ levels were significantly reduced in the severe malaria group, compared with those in the mild malaria group (P = .018; figure 1).

Although acetylaminoephene is thought to have no effect on systemic (non-central nervous system [CNS]) PG production, in previous studies of Gabonese children phytohemagglutinin-induced levels of TNF-α, and reactive oxygen species production were lower in children treated with acetylaminoephene [13]. Some children were given acetylaminoephene by their care givers before presentation for treatment. We compared bicyclo-PGE₂ levels in children given acetylaminoephene before being evaluated at the hospital with those who did not receive antipyretic treatment. There was no significant effect of acetylaminoephene treatment on plasma bicyclo-PGE₂ in children with mild malaria (acetylaminoephene vs. no acetylaminoephene, 18.6 ± 3.2 vs. 20.6 ± 2.6) or severe malaria (acetylaminoephene vs. no acetylaminoephene, 15.9 ± 3.9 vs. 15.0 ± 4.1).

**PBMC COX-2 protein and mRNA.** To determine whether altered COX-2 expression in leukocytes was correlated with the decreased PGE₂ production, COX-2 protein was determined in acutely isolated (noncultured) PBMC. This cell preparation was selected because leukocytes are an important source of PG during inflammation. COX-2 antigen expression in PBMC was inversely associated with disease severity (figure 2A is a representative immunoblot). PBMC COX-2 protein expression was quantified by densitometry. Relative to the healthy control group, COX-2 antigen expression was significantly lower in the mild (P = .026) and severe malaria groups (P < .01; figure 2B).

In a subset of PBMC samples from each of the 3 groups, we also performed reverse transcription–PCR, to determine COX-2 mRNA expression. Consistent with the results of the bicyclo-PGE₂ analysis and the COX-2 immunoblot results, COX-2 mRNA expression was inversely associated with disease severity (figure 2C is a representative gel). COX-2 mRNA expression in PBMC extracts was detected in 4 of 4 healthy control subjects, 1 of 4 patients with mild malaria, and 0 of the 4 children with severe malaria.

**Plasma cytokine association with plasma bicyclo-PGE₂.** Since a lower level of COX-2 gene expression in the mild and severe malaria groups could result from an inadequate proinflammatory response or from overexpression of anti-inflammatory cytokines, we measured plasma concentrations of IFN-γ, TNF-α, and IL-10. Relative to the control group, plasma levels of IFN-γ and TNF-α were significantly higher in the mild and severe malaria groups (figure 3A and 3B). However, plasma bicyclo-PGE₂ levels did not significantly correlate with plasma IFN-γ (r = .186; P = .124; r = .23). Plasma IL-10 levels were significantly higher in children with mild and severe malaria, compared with those of healthy donors (figure 3C). Consistent with the notion that anti-inflammatory cytokines decrease COX-2 gene expression [5], plasma bicyclo-PGE₂ showed a statistically significant inverse association with IL-10 (r = .38; P < .01; r = .38; figure 3D). These results suggest that elevated levels of IL-10 may decrease COX-2 expression and may

<table>
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<tr>
<th>Variable</th>
<th>Mild malaria (n = 54)</th>
<th>Severe malaria (n = 50)</th>
<th>P</th>
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<tr>
<td>Age, years</td>
<td>3.5 (0.4)</td>
<td>3.6 (0.7)</td>
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<td>Hyperparasitemia</td>
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<td>Hemoglobin (&lt;6.0 g/dL)</td>
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<td>0 (0)</td>
<td></td>
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<td>PCV (&lt;20%)</td>
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<td>0 (0)</td>
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<td>Schizotemia</td>
<td>0 (0)</td>
<td>0 (0)</td>
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<td>Parasitemia/µL</td>
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<td>333,772 (25,025)</td>
<td>.0001</td>
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<td>Geometric mean</td>
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<td>283,735</td>
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<tr>
<td>Median</td>
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<td>300,000</td>
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<tr>
<td>Hemoglobin, g/dL</td>
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<td>6.8 (0.3)</td>
<td>.0001</td>
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<tr>
<td>PCV</td>
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<td>21.9 (0.9)</td>
<td>.0001</td>
</tr>
<tr>
<td>Platelet count</td>
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<td>123.8 (9.1)</td>
<td>.0001</td>
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<td>Glucose, mg/dL</td>
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<td>79.8 (5.3)</td>
<td>.026</td>
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<tr>
<td>Admission temperature, °C</td>
<td>38.2 (0.3)</td>
<td>39.0 (0.2)</td>
<td>.001</td>
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NOTE: Data represent mean (±SE) unless otherwise stated. No subjects had cerebral malaria. PCV, packed cell volume.

* Inclusion criteria for severe cases: severe malaria was defined as parasitemias >250,000 parasites/µL, regardless of PCV, or parasitemias >100,000 parasites/µL in the presence of severe anemia (hemoglobin <6.0 g/dL or PCV ≤ 20%).
promote a subsequent decline in blood bicyclo-PGE₂ in malaria-infected children.

Discussion

PGs are important soluble mediators involved in the immune response to invading pathogens. COX-2, the major COX isoform in leukocytes, is responsible for the production and regulation of high levels of PG during an inflammatory event [5]. Although much is known regarding the molecular and cellular regulation of COX-2 and PG, the role of PG in regulating the immune response in malaria (and other human infectious diseases) is not fully understood. In our studies of Gabonese children with falciparum malaria, we demonstrated a significant reduction of plasma bicyclo-PGE₂ and PBMC cyclooxygenase-2 in children with mild malaria and an even more pronounced decrease in those with severe malaria. In addition, plasma IFN-γ, TNF-α, and IL-10 increased with disease severity, and IL-10 had a significant inverse correlation with plasma bicyclo-PGE₂.

PG produced during inflammation appeared to promote immunosuppression; PGE₂ can inhibit lymphocyte proliferation, cytokine production, migration, and cell-mediated cytotoxicity (reviewed in [7]). In murine models of malaria, enhanced production of PG results in decreased lymphocyte mitogenesis [18]. Clark and Hunt [19] postulated that an increase in arachidonate metabolites in peritoneal cells from mice infected with *Plasmodium vinckei vinckei* elicits the immunosuppression observed in this disease model. Studies in mice show that infection with *Leishmania* organisms causes increased PG production that suppresses cell-mediated immunity, at least in part, by inhibiting the development of a Th1 response [20]. Our finding of reduced plasma levels of PGE₂ and PBMC COX-2 expression in children with *P. falciparum* suggests that human malarial infections cause decreased production of monocyte-derived PG. This decreased PG production could be proinflammatory, since PGE₂ is a potent inhibitor of TNF-α production by monocytes [21]. Also, inhibition of PG synthesis in human volunteers injected with endotoxin is associated with elevated plasma TNF-α [10]. Results presented here that show low levels of plasma PGE₂ and PBMC COX-2 expression in the presence of high levels of TNF-α parallel previous observations and are consistent with the notion that monocyte-derived PGE₂ may regulate TNF-α production. A decrease in the ability of monocytes to produce PGE₂ may result in the overproduction of TNF-α. Elevated TNF-α levels are associated with severe malarial anemia [22], the primary clinical manifestation of severe disease in our study population.

Since fever is a hallmark clinical feature of malaria, one might expect increased systemic (non-CNS) production of PG. However, systemic production of PGE₂ during inflammation is probably regulated differently than PGE₂ produced within the brain for the generation of the febrile response. PGE₂ in venous blood is completely inactivated during passage through the lungs, and the concentration of PGE₂ delivered to the brain by arterial blood is low [23]. The systemic determinants of fever appear to be proinflammatory cytokines, such as IL-1 and TNF, that migrate to the preoptic area of the anterior hypothalamus, to regulate the febrile response by promoting production of PGE₂.
Figure 3. Pro- and anti-inflammatory cytokines and bicyclo-prostaglandin E$_2$ (PGE$_2$) in healthy children (control [Con]), children with mild malaria (MM), and children with severe malaria (SM). A–C, Data are mean ± SE. A, Interferon (IFN)–γ. Con vs. MM, $P < .01$; Con vs. SM, $P < .001$; and MM vs. SM, $P = $ not significant (NS). B, Tumor necrosis factor (TNF)–α. Con vs. MM, $P < .03$; Con vs. SM, $P < .001$; and MM vs. SM, $P = $ NS. C, Interleukin (IL)–10. Con vs. MM, $P < .01$; Con vs. SM, $P < .001$; and MM vs. SM, $P < .03$. D, Plasma bicyclo-PGE$_2$, in relation to plasma IL-10 levels.

from brain microvascular endothelium and/or astrocytes [24]. Peripheral participation in the febrile response may, therefore, involve cytokines (not PG, per se) produced outside the CNS. This may in part explain our observation that Gabonese children infected with *P. falciparum* have high plasma TNF–α levels and low plasma levels of PGE$_2$, in the presence of fever. Thus, on the basis of results presented here and on previous observations, we propose that low systemic levels of PGE$_2$ in children with malaria could augment TNF production and contribute to fever. Although recent studies have noted that *P. falciparum* can produce PG [25], it is unlikely that parasite-derived production of PG contributed to our observations, since PG levels were actually lower with increasing parasite burden.

Host resistance to malaria is promoted by the release of proinflammatory cytokines, such as IL-12, IFN–γ, and TNF–α, whereas anti-inflammatory cytokines, such as IL-10 and transforming growth factor–β counterregulate the proinflammatory response and may reduce resistance to disease [26, 27]. COX-2 expression is generally increased by proinflammatory cytokines and suppressed by anti-inflammatory cytokines [5]. The relative balance of pro- and anti-inflammatory cytokines, released as part of an inflammatory event, can regulate high-level output of PG by modulating expression of COX-2. Our results show that proinflammatory (IFN–γ and TNF–α) and anti-inflammatory (IL-10) cytokines increase with disease severity in this population of children. However, the statistically significant inverse correlation between bicyclo-PGE$_2$ and plasma IL-10 suggests that IL-10 may be responsible for the low levels of COX-2 expression in PBMC from these children who develop high-density parasitemia and severe malarial anemia. Additional studies that examine the role of PG in cerebral malaria may help to further determine whether reductions in COX-2 and PGE$_2$ are central to the pathogenesis of malaria. Future studies of COX-2 and PG in malaria should provide valuable information regarding disease pathogenesis and may provide insight into the development of new treatments for this global disease.
Acknowledgments

We thank the staff of Albert Schweitzer Hospital for cooperation and technical assistance: Anita van den Biggelaar, Judith Jans, Hanna Knoop, Doris Luckner, Barbara Moritz, Anselme Ndzeugue, Marcel, Nkeyi, Daniela Schmid, and Milena Sovric; Mary Misukonis (Veterans Affairs Medical Center, Durham, NC) for technical assistance; Nick Anstey (Menzies School for Health Research, Australia) for careful review of the manuscript; and James B. Hittner (Department of Psychology, College of Charleston, SC) for statistical expertise and critical evaluation of the manuscript.

References