ROLE OF EICOSANOIDS IN THE PATHOGENESIS OF MURINE CEREBRAL MALARIA

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Abstract. Because microvascular damage is a common feature of both primate and murine cerebral malaria, we have examined the role of eicosanoid metabolites (prostaglandins and leukotrienes) in experimental cerebral malaria. Eighty ICR mice were infected with Plasmodium berghei ANKA, with 40 uninfected mice as controls. Half of the infected mice were treated on days 4 and 5 with aspirin, a prostaglandin synthesis inhibitor. Infected mice started to die of cerebral malaria on day 6, and by day 17, all infected mice died. In contrast, all infected mice treated with aspirin died by day 12. Infected mice had increased phospholipase A2 mRNA expression in the spleen and cyclooxygenase 1 (COX1) and COX2 expression in the brain. At the peak of cerebral malaria, infected mice had higher serum leukotriene B4 levels than control mice, and aspirin-treated infected mice had higher serum leukotriene B4 levels than untreated infected mice. These results suggest that prostaglandins are protective whereas leukotrienes are detrimental in cerebral malaria.

Microvascular damage is a common feature of both primate and murine cerebral malaria. Humans who die of cerebral malaria caused by Plasmodium falciparum infection have petechial and ring hemorrhage and edema in the brain.1–6 Similar findings are also seen in cerebral malaria of rhesus monkeys caused by P. coatneyi and of mice caused by P. berghei ANKA.7 The mechanisms involved in the induction of microvascular damage during cerebral malaria are not well studied. Because cerebral malaria is associated with the over-production of T helper-1 and proinflammatory cytokines (tumor necrosis factor-α [TNF-α], interferon-γ [IFN-γ], and interleukin-1 [IL-1]),8–12 it is suggested that these cytokines cause cerebral malaria by up-regulation of intercellular adhesion molecule-1 (a cytoadhesion molecule involved in the adhesion of parasitized erythrocytes and mononuclear cells to endothelial cells) expression and nitric oxide production, which lead to mechanic blockage and disrupted neurotransmission.11–13 However, whether endothelial blockage and activation themselves cause brain hemorrhage is not clear. The role of platelets in cerebral malaria has attracted some attention, although detailed pathways for the platelet-induced pathology have not yet been studied.14

An alternative effect of the proinflammatory cytokines is the modulation on the production of eicosanoids (prostaglandins and leukotrienes), which are important in the maintenance of the structure and function of blood vessels.15 Increased phospholipase A2 activities have been reported in adults infected with P. falciparum.16 Children with cerebral malaria also have higher phospholipase A2 expression than those with uncomplicated malaria.17 Because the expression of other enzymes such as cyclooxygenase 1 and 2 (COX1 and COX2) (for prostaglandin synthesis) and lipooxygenase (for leukotriene synthesis) in malaria infection is not known, it is not clear which eicosanoid pathway is involved in the pathogenesis. Malaria infection is known to increase the production of prostaglandins and thromboxane B2 by monocytes.18 This increased production of prostaglandins is associated with the immunosuppression caused by malaria.19,20 The immunosuppression induced by prostaglandins may be protective rather than detrimental in cerebral malaria. This is supported by an anecdotal report, in which improvement of symptoms in a cerebral malaria patient was shown after treatment with prostacyclin (prostaglandin I2).21 Treatment with iloprost, a prostacyclin analog, also prevents the development of cerebral malaria in CBA/Ca mice infected with P. berghei ANKA.22 The role of leukotrienes and other hydroxy acids in the pathogenesis of cerebral malaria has not been examined, although we have previously shown that malaria pigment can catalyze the formation of 5-, 12-, and 15-hydroxyicosatetraenoic acids from arachidonic acids.23

In this study, we have examined the role of eicosanoids in the pathogenesis of malaria infection. The expression of rate-limiting enzymes involved in eicosanoid metabolism was characterized in mice infected with P. berghei ANKA. The role of prostaglandins was further evaluated by treatment of infected mice with aspirin, a salicylate that inhibits the activity of COX. Results of the study suggest that prostaglandins are protective whereas leukotrienes are detrimental in the pathogenesis of murine cerebral malaria.

MATERIALS AND METHODS

Experimental design. One hundred twenty 6–8-week-old female ICR mice were allotted to one of the three groups, each with 40 animals: an uninfected control group, an infected group, and an infected-treated group. Mice in the infected and infected-treated groups were each inoculated intraperitoneally with 2 × 106 P. berghei ANKA-infected erythrocytes. Mice in the infected-treated group were further treated intraperitoneally with 50 mg/kg of aspirin twice a day on days 4 and 5. Four mice from each group were killed 2, 7, and 12 days after infection. Samples were taken from the brain and spleen for total RNA extraction after peripheral blood (1–1.5 ml per mouse) was drained. Parasitemia of all infected animals were also determined simultaneously. Laboratory animal use protocols were reviewed and approved by the Centers for Disease Control and Prevention Institutional Animal Care and Use Committee.

Primers and probes. Oligonucleotides were synthesized at the Biotechnology Core Facility, Centers for Disease Control and Prevention (Atlanta, GA). All 5′ primers were conjugated with biotin, whereas all probes were labeled with digoxigenin, using 5′ labeling techniques standardized in the laboratory. The sequences of primers and probes used are shown in Table 1.

Quantitative reverse transcription–polymerase chain reaction (RT-PCR). Messenger RNA expression of major enzymes involved in eicosanoid metabolism was quantitated

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by a bioluminescence immunoassay-based RT-PCR. Total RNA was extracted from the spleen and brain using Ultrapure (Biotecx Laboratories, Houston, TX). Five micrograms of total RNA from each sample was used to synthesize cDNA, using the Superscript Preamplification System (Gibco-BRL, Gaithersburg, MD). One-tenth of the cDNA reaction mixture was added to a PCR mixture containing 100 nM biotinylated 5' primer, 100 nM 3' primer, 200 μM each of deoxynucleotide triphosphate, 1× PCR buffer (Perkin-Elmer Cetus, Norwalk, CT). Each cycle consisted of denaturation at 94°C for 5 min, 30–35 cycles of PCR were performed in the GeneAmp 9600 (Perkin-Elmer Cetus, Norwalk, CT). Each cycle consisted of denaturation at 94°C for 45 sec, annealing at 53°C for 45 sec, and extension at 72°C for 45 sec, with a final extension at 72°C for 10 min. The PCR-amplified products were captured onto microtiter plates coated with streptavidin after hybridization with digoxigenin-labeled probes. Aequorin-conjugated anti-digoxigenin antibodies were added to the plates. After washing off the excess antibodies, the aequorin-generated luminescence was triggered by the addition of calcium and measured with an MLX luminometer (Dynatech Inc., Chantilly, VA). The amount of specific mRNA present in samples was expressed as relative light units (RLU) obtained from the bioluminescence immunoassay. To control the amount of mRNA used in cDNA synthesis, β-actin was amplified from each cDNA synthesis.

**Leukotriene B4 measurement.** Serum leukotriene B4 levels of four animals from each group were determined at day 7, using a commercial enzymatic immunoassay kit (Cayman Chemicals, Ann Arbor, MI). After the serum samples were passed through Sep-Pak C18 cartridges (Waters, Milford, MA) for solid-phase extraction.

**Results.**

**Parasitemia and survival.** Control animals had no parasites in their peripheral blood during the study (Figure 1A). Mice in the infected and infected-treated groups also had no parasitemia at day 2. Parasitemia gradually increased in the infected and aspirin-treated groups afterwards. Parasitemia in the aspirin-treated group was slightly higher at day 12 than in the infected group due to the low number of survived mice in the aspirin group. No mice in the uninfected control group died during the experiment. Mice in the infected and aspirin treated groups started to die of malaria five days after the infection (Figure 1B), with animals in the aspirin-treated groups dying quicker than those in the infected group. By days 12 and 17, all animals in the aspirin-treated group and infected groups died from the infection, respectively. Differences in the survival between the infected and aspirin-treated groups were significant at days 6, 7, 8, and 12 (P ≤ 0.05).

**Expression of phospholipase A2.** The expression of phospholipase A2 in the spleen was maintained at low levels in uninfected control animals through the experiment (Figure 2A). Infection with *P. berghei,* however, increased the expression substantially. This increased expression was obvious even at day 2 before the increase in parasitemia. Increases in phospholipase A2 expression continued at days 7 and 12, and reached the maximum at day 16 when all infected animals died. Infected animals treated with aspirin also had increased expression of phospholipase A2 in the spleen (Figure 2A). They also had higher phospholipase A2 expression than infected mice at day 12.

The expression of phospholipase A2 in the brain was much different from in the spleen (Figure 2B). Constitutive expression of phospholipase A2 in the brain was much higher than in the spleen. Furthermore, *P. berghei* infection failed to up-regulate the high constitutive expression of
phospholipase A2. Aspirin treatment also had no effect on the expression of phospholipase A2 in infected animals.

Expression of COX1. Expression of COX1 in the spleen was similar among the three groups of animals (Figure 3A). Animals infected with *P. berghei* had COX1 expression slightly higher at day 2 and lower at day 12 than uninfected controls, but these differences were not significant. Aspirin treatment also had no significant effect on the expression of COX1.

*Plasmodium berghei* infection also had no significant effect on the expression of COX1 in the brain most of the time (Figure 3B). Compared with the uninfected controls, the expression in the infected animals was higher at day 7 ($P \leq 0.05$). Aspirin treatment reduced malaria-up-regulated COX1 expression in the brain at day 7.

Expression of COX2. There were no significant changes in COX2 expression in the spleen among the three groups during the experiment (Figure 4A). Expression of COX2 in the brain also stayed the same level in the uninfected control animals at all sampling date (Figure 4B). *Plasmodium berghei*-infected mice also had COX2 expression similar to the uninfected controls at day 2. However, they had increased COX2 expression at day 7 ($P \leq 0.05$). Aspirin treatment reduced malaria-induced COX2 expression in infected animals.

Expression of 5-lipoxygenase. Expression of 5-lipoxygenase in the spleen and brain remained similar between the uninfected control animals and mice infected with *P. berghei* (Figure 5). Aspirin treatment also had no significant effect on the expression of 5-lipoxygenase.

Production of leukotriene B4. To determine whether increased phospholipase A2 expression and the aspirin treatment would lead to increased production of leukotrienes, we measured serum levels of leukotriene B4 of mice at the peak of the occurrence of cerebral malaria (day 7 of the infection). As expected, *P. berghei* infection up-regulated leukotriene
DISCUSSION

Results of this study indicate that malaria modulates the expression of enzymes involved in eicosanoid metabolism. *Plasmodium berghei*-infected mice had increased expression of phospholipase A2 in the spleen and COX1 and COX2 in the brain. In line with the increased expression of phospholipase A2, serum levels of leukotriene B4 were also increased in *P. berghei*-infected mice. The increased expression of phospholipase A2 in the spleen is in agreement with results of previous studies, in which *P. falciparum*-infected patients were shown to have increased phospholipase A2 activities in the peripheral circulation.\(^{16,17}\) Increased COX1 and COX2 expression in the brain but not the spleen of infected animals indicates the presence of variations in the expression of eicosanoid enzymes in different organs.

The up-regulation in the expression of phospholipase A2, COX1, and COX2 enzymes is likely to be the result of increased production of proinflammatory cytokines such as TNF-\(\alpha\), IFN-\(\gamma\), and IL-1\(\beta\) in malaria. It is well established that the production of these cytokines is increased in murine cerebral malaria.\(^{10}\) Recent *in vitro* studies have also shown that the expression of some enzymes involved in eicosanoid metabolism is affected by various cytokines.\(^{25}\) Both TNF-\(\alpha\) and IL-1 increase the expression of phospholipase A2 and COX2 in various culture cells.\(^{15}\) In contrast, the expression of COX1 is constitutive and less affected by cytokines. Although proinflammatory cytokines (TNF-\(\alpha\) and IL-1) and other stimuli can increase the activities of 5-lipoxygenase, this effect is largely due to the increased release of the enzyme from cytosol rather than increased production upon activation.\(^{15}\)
The increase in eicosanoid enzyme activities may play a role in the pathogenesis of cerebral malaria. The increased expression of eicosanoid enzymes at day 7 of the infection coincided with the peak occurrence of cerebral malaria.\(^9\)\(^{-11}\) This increased production of phospholipase A2 and release of 5-lipoxygenase upon immune activation by malaria infection can result in the increased synthesis of leukotrienes and other hydroxy acids. Because the latter cause leukocyte adhesion and activation, increased vascular permeability, and production of proinflammatory cytokines,\(^{15}\) it is likely that they will promote the pathogenesis of cerebral malaria. On the other hand, the increased release of arachidonic acid after immune activation can lead to the increased production of prostaglandins under the reaction of COX, which cause immunosuppression, reduced leukocyte adhesion, decreased cytokine excretion, and vasoprotection. The latter in turn can prevent much of the hyperimmune responsiveness associated with cerebral malaria.

In agreement with our observations, Sliwa and others have previously shown that treatment with a prostacyclin analog can prevent the occurrence of cerebral malaria in mice infected with *P. berghei* ANKA.\(^{22}\) Treatment with dietary fish-oil supplementation, which interferes with eicosanoid production, also suppresses the occurrence of cerebral malaria in mice.\(^{26}\) Thus, the occurrence of cerebral malaria may well be a balance between the production of immunostimulative leukotrienes and the immunosuppressive prostaglandins.

Results of treatment of *P. berghei*-infected mice with the prostaglandin synthesis inhibitor aspirin support the role of eicosanoids in the pathogenesis of cerebral malaria. Rapid induction of cerebral malaria was achieved by aspirin treatment of infected mice. Because aspirin inactivates COX1 and COX2 by acetylation, it is possible that synthesis of the prostaglandins were reduced in infected mice treated with aspirin. Furthermore, more arachidonic acids were probably diverted to the synthesis of leukotrienes, as indicated by the increased production of leukotriene B4 in aspirin-treated mice, thus aggravating the diseases. The increased leukotriene production and reduced prostaglandin synthesis probably contributed to the rapid occurrence of cerebral malaria in
aspirin-treated mice. This further supports the role of eicosanoids in the induction of immune activation and brain hemorrhage in cerebral malaria. Additional studies with other prostaglandin inhibitors are needed to fully address this issue.

The rapid occurrence of cerebral malaria in *P. berghei*-infected mice after aspirin treatment may explain one clinical observation made by English and others, in which it was shown that children with cerebral malaria who were treated with salicylates had poorer outcomes than those who received no salicylates such as aspirin. They attributed the increased mortality in salicylate-treated children to salicylate toxicity. In our study, mice were treated with a dose more than 20 times below the 50% lethal dose. Results of our study indicate that increased production of leukotrienes after aspirin treatment may also play an important role in the increased mortality in children with cerebral malaria.

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