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Antimalarial Properties of n-3 and n-6 Polyunsaturated Fatty Acids: In Vitro Effects on Plasmodium falciparum and In Vivo Effects on P. berghei

Lakshmi M. Kumaratilake,* Brenton S. Robinson,§ Antonio Ferrante,* and Alf Poulos†
*Department of Immunology and University of Adelaide Department of Paediatrics; and †Department of Chemical Pathology, Adelaide Children’s Hospital, South Australia 5006, Australia

Abstract
The polyunsaturated fatty acids docosahexaenoic acid (C22:6,n3), eicosapentaenoic acid, arachidonic acid, and linoleic acid caused marked in vitro growth inhibition of Plasmodium falciparum, assessed by a radiometric assay. In contrast, negligible parasite killing was seen with oleic acid or docosanoic acid. Parasite killing was significantly increased when oxidized forms of polyunsaturated fatty acids were used. Antioxidants greatly reduced the fatty acid–induced killing. Mice infected with P. berghei and treated for 4 d with C22:6,n3 showed marked reduction in parasitemia. The anemia associated with the infection was also alleviated by treatment with C22:6,n3. The data provide new information that could be explored in order to develop new strategies in malaria treatment. (J. Clin. Invest. 1992. 89:961–967.) Key words: anemia • antioxidants • crisis forms • fish oil • parasite growth inhibition

Introduction
Over half of the world’s population is at risk from malaria, involving some 100 countries, 500 million acute infections, and over 1 million deaths recorded per year (1, 2). As a whole, this situation has shown little improvement in the last 15 years. Vaccine development is proving lengthy and complex, and drug development continues to be a major goal in that the toxicity-related side effects of present chemotherapy render it only partially satisfactory and drug resistance is rife.

In the hope of developing unique strategies for the treatment of malaria, some investigations have concentrated on attempts to identify the natural molecules responsible for “crisis” formation (deranged development and degeneration of intrarheocytic malarial parasites; references 3–10). However, crisis-inducing factors still remain ill defined (11). This paper describes the in vitro killing of intrarheocytic forms of Plasmodium falciparum and the in vivo killing of P. berghei in mice by polyunsaturated fatty acids. The finding has the potential to be exploited for therapeutic purposes and may help explain mechanisms of crisis formation in vivo. Some of these polyunsaturated fatty acids are released by phagocyte leucocytes and/or may be acquired through normal diet or dietary supplements.

Methods
Parasite. Six isolates of P. falciparum were maintained under in vitro conditions in group O human red blood cells (RBC) in RPMI 1640 medium (CytoSystem Pty. Ltd., Sydney, Australia) supplemented with 10% heat-inactivated group AB+ serum, as described previously (12, 13). Experiments were performed with synchronized and nonsynchronized cultures at 108 RBC/ml with 3–5% parasitemia.

Mice. Female 6–8-wk-old BALB/c mice were used. They were fed with industrial mouse pellets (Milling Industries, Adelaide, South Australia) and water ad lib.

Fatty acids. Docosahexaenoic acid (C22:6,n3), docosahexaenoic acid methyl ester (C22:6,n3 methyl ester), eicosapentaenoic acid (C20:5,n3), arachidonic acid (C20:4,n6), linoleic acid (C18:2,n6), oleic acid (C18:1,n9), docosanoic acid (C22:0), and d-ta-Dipalmitoyl phosphatidylycholine (DPPC) were obtained from Sigma Chemical Co. (St. Louis, MO). Lipids were dissolved in redistilled chloroform (10–50 mg/ml) and stored under N2 at −20ºC. Thin-layer chromatography indicated that the lipids were of high purity. Mixed fatty acid–DPPC micelles were prepared as follows. Fatty acid (8, 32, or 80 µg) and DPPC (fourfold the amount of fatty acid) were dispensed into 10-ml glass tubes and the solvent was evaporated under N2. 2 ml of RPMI 1640 medium was added and the mixture was sonicated for 2 min with an ultrasonic processor (model W-225 ; Heat Systems-Ultrasonics, Inc., Farmingdale, NY) with 40% duty cycle and output setting 4. The lipid sonicates were clear under these conditions. 50-µl aliquots of each sonicate (containing 4, 16, or 40 µg/ml fatty acid) were used for assays unless stated otherwise.

Growth inhibition assay. This was carried out as described previously (12, 13) with the following modifications. Experiments were conducted in 96-well flat-bottomed microdilution plates (Linbro, Flow Laboratories, Inc., McLean, VA), comparing three different treatment groups. 50 µl P. falciparum–infected RBC were mixed with 50 µl of either fatty acids (40 µg/ml), diluent (DPPC), or malaria medium. The plates were incubated for 2 h at 37ºC in 5% CO2 in air. Then the wells were pulsed with 1 µCi of [3H]hypoxanthine (Amersham Corp., Arlington Heights, IL) in 50 µl of RPMI 1640 medium and incubated for a further 18 h at 37ºC in 5% CO2 in air. Individual well contents were then collected using a semiautomated sample harvester (Titerette, Flow Laboratories, Sydney, Australia) and the [3H]hypoxanthine incorporation was measured in a liquid scintillation counter (model LS 3801; Beckman Instruments, Carlsbad, CA). Percent growth inhibition of the parasite was calculated by the following formula: percent inhibition = [(dpm of parasite in diluent) – (dpm of parasite incubated with fatty

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1. Abbreviations used in this paper: BHT, butylated hydroxytoluene; C18:1,n-9, oleic acid; C18:2,n-6, linoleic acid; C20:4,n-6, arachidonic acid; C20:5,n-3, eicosapentaenoic acid; C22:0, docosanoic acid; C22:6,n-3, docosahexaenoic acid; DPPC, d-ta-Dipalmitoyl phosphatidylycholine; RBC and PRBC, red blood cells and parasitized RBC, respectively; TNFα, tumor necrosis factor α.

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Antimalarial Properties of Polyunsaturated Fatty Acids
Results

Effects of polyunsaturated fatty acids on P. falciparum in vitro. Fig. 1 summarizes the effects of fatty acids on the growth of sexual blood stages of P. falciparum. Treatment of parasites with C22:6n-3, C20:5n-3, C20:4n-6, and C18:2n-6 resulted in marked inhibition of P. falciparum growth. However, the fatty acids C18:1n-9 and C22:6n-3 had very little effect. The highest antimalarial activity was always seen with C22:6n-3 (76±3.3% growth inhibition). The methyl ester of this fatty acid was just as effective (71±4.3%). Similar results were obtained when fatty acids were delivered in either albumin or DPPC (data not presented). The antimalarial activity of the fatty acids was concentration dependent (Fig. 2). Within the range of 4 to 40 μg/ml, the fatty acids did not induce hemolysis of parasitized or normal RBC as assessed by hemoglobin release and by morphological criteria. At these concentrations, DPPC was not toxic to P. falciparum and [3H]hypoxanthine incorporation was not affected by the presence of the phospholipid.

Microscopic examination of fatty acid–treated P. falciparum. Microscopic examination showed that parasites exposed to polyunsaturated fatty acids for 18 h underwent degener-
eration and death (Fig. 3). We found that immature and mature schizonts were more susceptible to the fatty acid–induced effects than were ring forms. However, the majority of ring forms were also found to be degenerated. The dead parasites were removed from the parasitophorous vacuole through its orifice to the exterior of the RBC. The fatty acid C\textsubscript{22,6,n-3} caused >90% death of \textit{P. falciparum}. The survived parasites were in the ring form. C\textsubscript{20,4,n-6} caused 80% death or degeneration of the parasite. In addition to rings, a small percentage of schizonts also survived when treated with C\textsubscript{20,4,n-6}. In contrast, C\textsubscript{22,0}–treated \textit{P. falciparum} cultures were very similar to the parasite grown in the absence of fatty acids.

\textbf{Effects of fish oil.} Fish oil compared with 40 \textmu g/ml C\textsubscript{22,6,n-3} showed a much lower inhibition of the parasite growth. When PRBC were treated with 40 \textmu g/ml of fish oil for 2 h, the parasite inhibition was 20.5±8.5%. However, when the interaction time was increased to 20 h, more parasite inhibition (41.0±5.5%) was observed.

\textbf{Effects of oxidized fatty acids.} The oxidized forms of these fatty acids showed increased activity against \textit{P. falciparum} (Fig. 4). Oxidized C\textsubscript{22,6,n-3} and C\textsubscript{20,4,n-4} showed significantly more antiparasitic activity (\(P<0.01\)) than the nonoxidized forms (Fig. 4). Antiparasitic effects were reduced by ~50% if PRBC had been preincubated with BHT or vitamin E overnight, washed, and then exposed to the native fatty acids. Antiparasitic activity was reduced by >70% when the preincubated PRBC were reacted with fatty acids in the presence of BHT and vitamin E (Fig. 4). The effects of polyunsaturated fatty acids were also greatly reduced if the PRBC were treated with SOD or catalase (Fig. 4). Ethanol (0.5%), the diluent for some of these reducing reagents, had no effect on parasite growth.

\textbf{Effects of polyunsaturated fatty acids on \textit{P. berghei} infection in mice.} Mice treated with the fatty acid vehicle DPPC showed parasitemias similar to those of mice that received saline (Fig. 5). The parasitemias increased steadily in these mice. 6 d after infection, these mice showed a 60-fold increase in parasitemia (Fig. 5). After a single injection of the fatty acid C\textsubscript{22,6,n-3}, the parasitemia was significantly reduced from 1.65±0.40 to 0.25±0.04% (\(P<0.001\); Fig. 5). Throughout the course of the treatment, the parasitemia was maintained at a lower level than the initial parasitemia (Fig. 5). Compared with the mice that received DPPC or saline, the fatty acid–treated mice showed markedly lower parasitemia (\(P<0.001\)). In the mice treated with DPPC or saline, the parasites grew rapidly, showing all different stages of the asexual and sexual stages. In contrast, in C\textsubscript{22,6,n-3}–treated mice, 70–100% of the surviving parasites had “restricted growth” and appeared as a “dot” form. C\textsubscript{20,4,n-6} was similarly effective in reducing the parasitemia, but C\textsubscript{22,0} had no effect (data not presented).

Mice were examined for anemia. The hematocrit values for DPPC–, saline–, and C\textsubscript{22,6,n-3}–treated groups were 26.3±2.3, 25.9±2.5, and 40.7±6.9, respectively.

\textbf{Discussion}

The data demonstrate that fatty acids vary in ability to cause growth inhibition of \textit{P. falciparum} in culture. Whereas the polyunsaturated fatty acids C\textsubscript{22,6,n-3}, C\textsubscript{20,5,n-3}, C\textsubscript{20,4,n-6}, and C\textsubscript{18,2,n-6} caused marked growth inhibition of the parasite, the monounsaturated and saturated fatty acids C\textsubscript{18,1,n-6} and C\textsubscript{22,0} had little effect. This result demonstrates that the antimalarial activity of the fatty acids is dependent in part on the degree of unsaturation (number of double bonds present). For example, the degree of inhibition induced by the 22-C fatty acids was increased severalfold by the introduction of six double bonds into the molecule. Similarly, the addition of a single double bond into
the monounsaturated fatty acid $C_{18:1,9}$ more than tripled the parasiticidal effects of the molecules.

At the concentrations tested, the fatty acids were not toxic for either normal RBC or PRBC and did not induce hemolysis (determined biochemically). Similar results were observed between the fatty acids solubilized in DPPC or albumin, indicating that binding of fatty acids to albumin in vivo is unlikely to inhibit their antimalarial activity.

Results of the in vivo experiments confirmed the antiplasmodial effects of polyunsaturated fatty acids. The fatty acids $C_{22:6,3}$ and $C_{20:4,6}$ were capable of inhibiting the growth of $P. berghei$ in mice, but the saturated fatty acid $C_{22:0}$ had no effect.
on *P. berghei*. Importantly, the fatty acids showed no toxic effects in mice but, in fact, prevented the malaria-induced anemia.

Morphological studies confirmed the results obtained from the radiometric assay and revealed that fatty acids caused intraerythrocytic degeneration and death of *P. falciparum*. Intraerythrocytic degenerative forms (crisis forms) of malarial parasites were observed as early as 1944 by Taliaferro and Taliaferro (17) in *P. brasilianum*-infected cebus and spider monkeys. Since then, abnormal forms of intraerythrocytic *Plasmodium* species have been reported in rodents, primates, and humans (for review see reference 11). Tumor necrosis factor α (TNFα; references 10, 18, 19), or a combination of TNFα and interferon γ with some undefined factors (5, 20), has been suggested as candidates for crisis-inducing factors. Under in vitro conditions, we have found no evidence that any of the macrophage and lymphocyte cytokines; TNFα; lymphotoxin; interferon γ; interleukins-1, -2, -4, or -8; or granulocyte macrophage colony-stimulating factor induce degeneration of intraerythrocytic *P. falciparum* in the presence or absence of complement and antimalarial antibody (12, 13, 21). Others have suggested a role for molecules such as oxygen-derived reactive species (4, 22), reactive nitrogen intermediates (23), oxidized polyamines (24), and lipid peroxides (4, 7). Our studies suggest that polyunsaturated fatty acids may constitute a crisis-inducing system in *P. falciparum*-infected RBC.

The mature mammalian RBC has negligible lipid metabolism, and *Plasmodia* cannot form fatty acids by de novo synthesis (25, 26). Under the influence of the parasite, host RBC cell membrane is grossly altered in structure and composition. Radiolabeled studies have shown that the uptake of certain fatty acids increases dramatically after the infection with malaria parasites (27–30). Interestingly, malaria infection causes a marked reduction in the unsaturation index of RBC phospholipids, mainly as a result of the decreases in the amounts of C12:0:3, C12:0:6, and C18:2:6 (27). Our study shows that when PRBC are exposed to the same fatty acids or their methyl esters or oxidized fatty acids, parasiticidal effects occur, although the basis for the toxicity probably differs according to the state of the fatty acid. Our morphological studies showed that the fatty acids induced death of the parasite and that later these dead

![Figure 4. The effects of oxidized fatty acids or "antioxidants." The effects of oxidation of the fatty acids or treatment of PRBC with antioxidants (Vit. E, vitamin E; BHT, SOD; and CAT, catalase) on the ability of the fatty acids to damage *P. falciparum*. Means±SEM are given.](image-url)

![Figure 5. The effect of C22:6:3 on *P. berghei* infection in mice. Mice were treated with C22:6:3 in DPPC (●), DPPC alone (●), or normal saline (*). Means±SEM are given.](image-url)
parasites move (via the parasitophorous vacuole orifice) to the exterior of the RBC. This observation suggests that the site of merozoite invasion, which acts as the orifice of the parasitophorous vacuole, has been damaged by the fatty acids or their derivatives.

It is evident from our studies that oxidized fatty acids are effective in inducing parasitoidal effects of *P. falciparum*. It is also likely that the effect of the parent nonoxidized fatty acid is related to fatty acid oxidation by the parasite or RBC, because antioxidants such as BHT and vitamin E markedly reduce the antimalarial activity of the fatty acids. Moreover, saturated and monounsaturated fatty acids, which are more resistant to oxidative attack, are much less effective than fatty acids with two or more double bonds. The nature of the oxidation products that induce the effect remains unclear. Lipid hydroperoxides (mainly mono- and dihydroxy derivatives) are major products of autoxidation, although a variety of other compounds (including alkanals, hydroxyalkanals, malonaldehyde, polymerized polar derivatives, and even volatile carbonyls) are produced (31). Whether some or all of these products are effective at inducing degeneration of the parasite remains to be determined. The mechanism of action of the active agents is clearly dependent on their structure. Thus, fatty acid hydroperoxides may act by oxidizing reduced glutathione and thereby making the parasite more susceptible to oxidative stress, whereas malonaldehyde is thought to interfere with membrane function through its ability to form cross-linkages between proteins and lipids (4). Recently it has been suggested that certain specific oxidized fatty acid products (e.g., oligomeric prostaglandins, which also inhibit the growth of *P. falciparum* [32]) could act through their ability to bind calcium or inhibited phospholipase or protease activities (33). Whether the oxidation products formed from the various polyunsaturated fatty acids used in our study act in a similar fashion remains to be established. Clearly, further studies with individual compounds are needed to clarify their action.

In vitro experiments of the present study showed that scavengers of reactive oxygen species and antioxidants reduced the fatty acid–induced killing of the parasite. It has been shown that administration of butylated hydroxyanisole, SOD, or catalase reduced cerebral pathology of *P. berghei* ANKA mice (34). Treatment of malaria-infected mice with these scavengers and antioxidants may counteract the fatty acid–mediated killing, if both treatments are given simultaneously. Treatment of mice with fatty acids alone, however, did not aggravate the pathology of malaria, probably related to the marked reduction of parasitemia.

Antimalarial activity of fish oil was relatively low. Fish oils are a source of triglycerides containing ω-3 fatty acids, and the preparation used in this study contained 18% C20:5n-3 and 12% C22:6n-3. It is possible that the remaining constituents of the fish oil counteracted the effects of C20:5n-3 and C22:6n-3. Alternatively, the triglycerides containing C16:0 and C18:2n-6 may not be available to the parasite, because of the relatively larger size of the molecule.

The data presented in this paper provide new strategies that can be explored for the treatment of malaria at a time when new antimalarials are in demand. These fatty acids, in addition to being able to act directly on the parasites, may also activate neutrophils (35) and other effector cells for increased killing of malarial parasites.

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**References**


