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Iron-Dependent Free Radical Generation from the Antimalarial Agent Artemisinin (Qinghaosu)

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Artemisinin is an important new antimalarial agent containing a bridged endoperoxide. The in vitro antimalarial activity of an artemisinin derivative, arteether, is antagonized by two iron chelators, pyridoxal benzoylhydrazone and 1,2-dimethyl-3-hydroxypyrid-4-one. Similarly, the acute toxicity of artemisinin in mice is antagonized by another chelator, deferoxamine-hydroxyethylstarch. A combination of artemisinin and hemin oxidizes erythrocyte membrane thiols in vitro, and this oxidation is also inhibited by an iron chelator. Thus, iron plays a role in the mechanisms of action and toxicity of artemisinin. The combination of artemisinin and hemin also decreases erythrocyte deformability. Iron probably catalyzes the generation of free radicals from artemisinin since α-tocopherol antagonizes the thiol-oxidizing activity of artemisinin and since a spin-trapped free radical signal can be seen by electron paramagnetic resonance only when artemisinin is incubated in the presence of iron.

Malaria remains an important global health problem, affecting an estimated 270 million people per year and killing 1 to 2 million people per year (47). Resistance to currently used antimalarial drugs such as chloroquine, quinine, mefloquine, and Fansidar is spreading rapidly (47). Thus, there is a great need for new antimalarial agents.

The antimalarial agent artemisinin (qinghaosu) was isolated in 1972 from *Artemisia annua*, an ancient Chinese herbal remedy for fever (for a review, see reference 26). Several artemisinin derivatives are currently undergoing phase I and II clinical studies, including artemether, arteether, and artesunate, which are the methyl ether, ethyl ether, and succinate esters, respectively, of dihydroartemisinin. Artemisinin and its derivatives have been widely used for the therapy of malaria in China, Vietnam, Thailand, and Myanmar; over 2 million doses of artemether have been administered in China alone. Artemisinin-type antimalarial agents are particularly useful against chloroquine-resistant *Plasmodium falciparum* strains and cerebral malaria (47).

The antimalarial action of artemisinin appears to be mediated by free radicals (27, 28, 31). The drug contains an endoperoxide bridge, which is necessary for biological activity (6, 8) because it reacts with intraparasitic heme iron (30). The reaction between artemisinin and heme accounts for the selectivity of the drug since high levels of heme are acquired by the parasite as a result of hemoglobin digestion (17). However, an artemisinin-derived free radical has hitherto never been demonstrated.

In this paper, we provide further evidence for the importance of iron in the mechanism of action of artemisinin and measure artemisinin-derived free radicals by electron paramagnetic resonance spectroscopy.

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MATERIALS AND METHODS

Abbreviations. The following abbreviations are used in this paper: DFO-HES, deferoxamine-hydroxyethylstarch conjugate; DMP, 1,2-dimethyl-3-hydroxypyrid-4-one; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; EC_{50}, the drug concentrations required to reduce incorporation by 50%; EPR, electron paramagnetic resonance; HES, hydroxyethylstarch; PBH, pyridoxal benzoylhydrazone.

Chemicals. Artemisinin and DMPO were purchased from Aldrich Chemical Company (Milwaukee, Wis.). Artemisinin was kept frozen (−70°C) as a 10 mM stock solution in ethanol. DMP and PBH were gifts from R. C. Hider and Prapin Wilairat, respectively. They were prepared as stock solutions of 0.4 M in sterile distilled water and 0.6 M in dimethyl sulfoxide, respectively. Arteether was a gift from the World Bank-United Nations Development Program-World Health Organization Special Program for Research and Training in Tropical Diseases and was prepared as a 90 mM stock solution. DFO-HES in 0.9% saline was a gift from Biomedical Frontiers, Minneapolis, Minn. 10% HES in 0.9% saline, also from Biomedical Frontiers, was used as a control. The DFO-HES solution contained the equivalent of 17 mM DFO. All other chemicals were purchased from Sigma Chemical Company (St. Louis, Mo.). A 1 mM hemin stock solution was made immediately prior to each experiment by first dissolving hemin in several drops of 1 M NaOH, diluting this solution with 1 M Tris, pH 8.0, and then bringing it to pH 8.0 with 1 M HCl.

In vitro antimalarial activity. In vitro antimalarial activity was tested by a modification of the method of Desjardins (11) using *P. falciparum* K1. Twenty-five microliters of medium containing drugs and 200 μl of a 1.5% infected-erythrocyte suspension (1 to 2% parasitemia) were placed in 96-well microtiter plates and cultured in a candle jar at 37°C. The final organic solvent concentration never exceeded 0.1%, a concentration which had no effect on parasite growth. After

24 h, 0.5-μCi [^{3}H]hypoxanthine was added to each well, and the mixture was cultured under the same conditions for an additional 18 h. The microtiter plates were harvested and counted as described previously (11), and the arteether EC_{50}s for various chelator concentrations and the chelator EC_{50}s for various arteether concentrations were determined. Iron-saturated DMP was prepared by incubating the chelator with ferric chloride (molar ratio of 3:1) at pH 2.0 for 15 min, followed by adjustment to pH 7.4 with sodium bicarbonate.

Effects of chelator on mouse survival. Groups of eight Swiss Webster male mice (22 g; Taconic Farms) were injected intraperitoneally with 0.1 ml of either DFO-HES or HES at the initiation of the experiment and again 24 h later. An artemisinin suspension was created by adding 200 to 300 mg of the drug to a mixture of 0.1% Tween and 0.5% HES and stirring the solution for 2 to 4 h. Four hours after the initial injection of DFO-HES or HES, the mice were injected with either 2.8 or 3.2 g of artemisinin per kg subcutaneously. Statistical significance was determined by the Wilcoxon signed-rank test (unpaired) with StatView II software (Abacus Concepts, Berkeley, Calif.).

Membrane thiol assay. Membranes were prepared from freshly obtained erythrocytes by the method of Dodge et al. (12). Suspensions of erythrocyte membranes were incubated at 37°C for 3 h in the presence and absence of artemisinin (1 mM) and/or hemin (100 μM) in sodium phosphate (14.3 mM phosphate, pH 7.4) buffer containing 10% ethanol. All reaction components were added at the beginning of each incubation except hemin, which was added after 1 h. Simultaneous incubations were performed in the absence of erythrocyte membranes, and these absorbances were subtracted from those measured in the presence of membranes. Membrane proteins were then solubilized by incubation for 10 min at 70°C in 2% sodium dodecyl sulfate. Thiol content was measured with a Bausch & Lomb Spectronic 2000 spectrophotometer by determining the difference in A_{412} before and 15 min after the addition of 20 μg of DTNB per ml (30, 46). Protein was determined by the method of Bradford (4).

Deformability studies. Erythrocyte deformability was measured with an Ektacytometer (Technicon Instruments, Tarrytown, N.Y.) coupled to a Macintosh computer (10). Prior to each deformability measurement, freshly drawn and washed erythrocytes were incubated at 37°C for 30 min in a 30% suspension in isotonic buffer (50 mM potassium phosphate, 0.9% sodium chloride) in the presence or absence of hemin and/or artemisinin.

EPR studies. EPR was performed on a JEOL/RE1X spectrometer with 100-kHz field modulation and Xband operation at 50 mV of power and with gain, modulation, and time constant set at 50, 0.32, and 0.03, respectively. g values were determined with a Mn^{2+} internal standard. Solutions containing 13 mM $Fe(NH_4)_2(SO_4)_2$, 13 mM artemisinin, and/or 10% (vol/vol) DMPO in methanol-water (85:15) were incubated at room temperature for 30 min prior to measuring spectra. Each scan took 8 min to perform.

RESULTS

Two chelators, which bind and sequester iron, antagonize the antimalarial effects of artemisinin. *P. falciparum*-infected erythrocytes were incubated in the presence of various concentrations of arteether, an artemisinin derivative, and either PBH or DMP. Since these iron chelators have their own inherent antimalarial activity, antagonism was assessed with isobolograms (3). For both chelators, the plots are strongly convex upwards, indicating that they antagonized arteether (Fig. 1). In contrast, iron-saturated DMP does not have this effect (Fig. 1B). Thus, iron appears to be important in the mechanism of action of artemisinin.

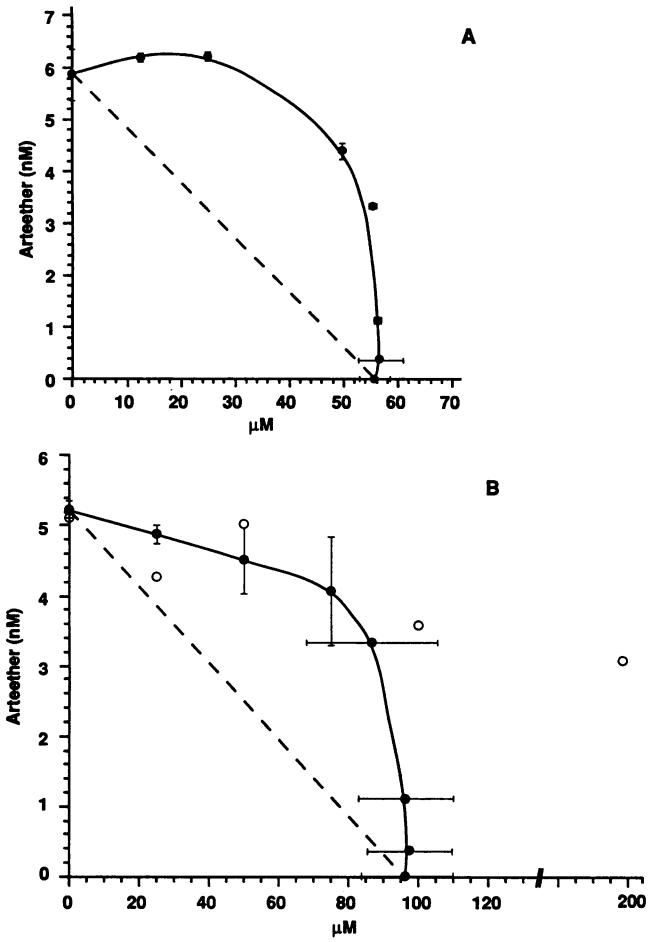

FIG. 1. Isobolograms of arteether in combination with the chelators PBH (A) and DMP (●) and iron-saturated DMP (○) (B). The error bars are ranges of EC_{50}s from two experiments, each of which was performed in triplicate. The error bars along the *y* axis are ranges of the arteether EC_{50} when the concentration of the chelator was fixed and that of arteether was varied. The error bars along the *x* axis are ranges of the chelator EC_{50} when the concentration of arteether was fixed and that of the chelator was varied.

Iron chelation also prevents the toxic effects of artemisinin in mice. This experiment was carried out with DFO-HES, a high-molecular-weight conjugate of DFO and HES, which was chosen for two reasons. First, because it has a long half-life, it can be administered on a daily basis (19). Second, because it is not taken up by *P. falciparum*-infected erythrocytes, it does not antagonize the antimalarial effects of artemisinin (unpublished data). Groups of eight mice were treated with high doses of artemisinin and either DFO-HES or HES (Fig. 2). Mice administered the higher dose of artemisinin (3.2 g/kg) and DFO-HES survived significantly longer than mice administered this dose of artemisinin and HES (13.9 versus 6.9 days, $P = 0.001$). Mice treated with the lower dose of artemisinin (2.8 g/kg) also survived significantly longer if they also received DFO-HES than did mice receiving this

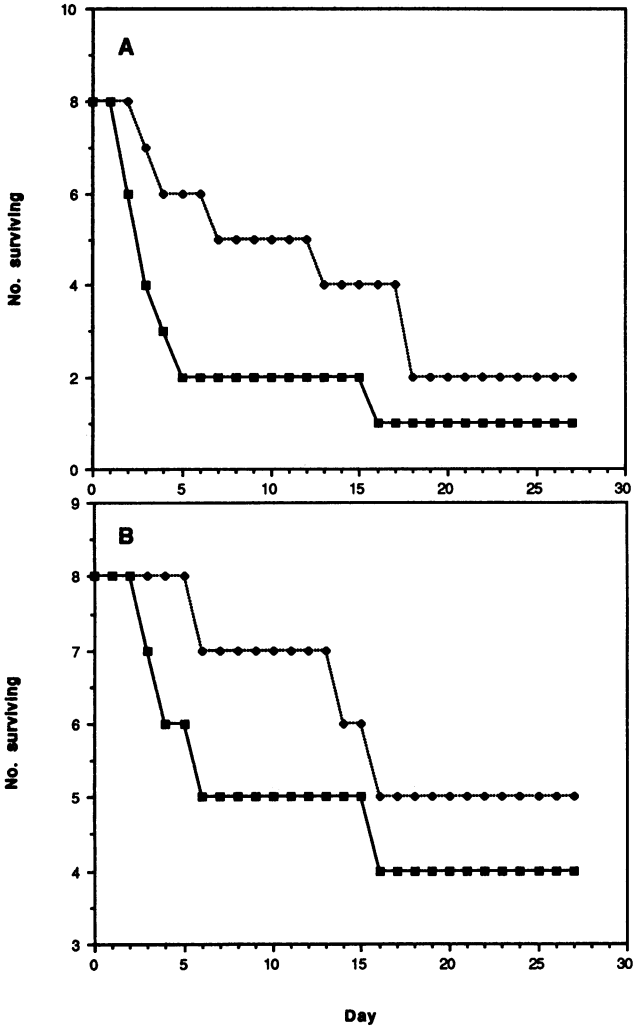

FIG. 2. Survival of uninfected mice after treatment with artemisinin and DFO-HES (◆) or HES alone (■). Survival was monitored for mice administered either 3.2 g/kg (A) or 2.8 g/kg (B).

dose of artemisinin and HES (21 versus 16.6 days, $P = 0.0004$). Thus, iron appears to play an important role in the toxic effects of high doses of artemisinin in animals.

The synergistic oxidation of erythrocyte membrane protein thiols by a combination of artemisinin and hemin (30) can be antagonized by an iron chelator and a free radical scavenger (Fig. 3). A combination of hemin and artemisinin oxidizes 88% of the membrane thiols, whereas artemisinin alone or hemin alone (data not shown) has little effect. In contrast, only 27% of membrane thiols are oxidized when the artemisinin-hemin-mediated oxidation is carried out in the presence of α-tocopherol, a free radical scavenger. Similarly, only 6% of them are oxidized when the reaction is carried out in the presence of the chelator DFO. This latter effect can be due to the sequestration of free iron by DFO or by the binding of DFO to hemin (2, 44). Thus, the artemisinin-hemin-mediated oxidation of erythrocyte membrane proteins is dependent on iron and mediated by free radicals.

We then investigated whether a combination of artemisinin and hemin would resemble other oxidant agents in causing the loss of erythrocyte deformability (42, 46). Arteminisin and hemin were found to have synergistic effects

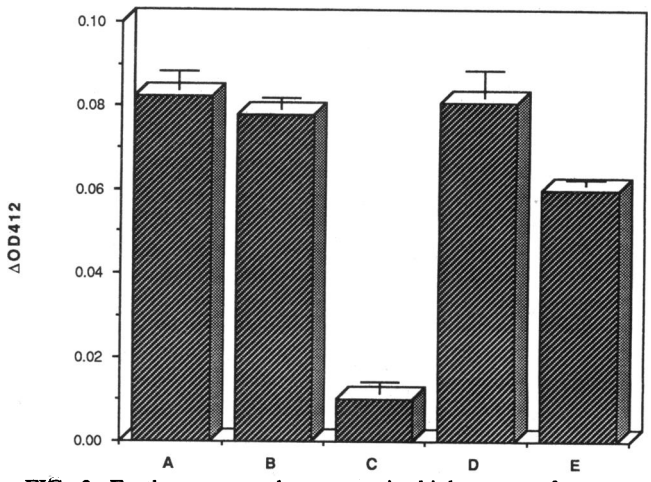

FIG. 3. Erythrocyte membrane protein thiol content after treatment with artemisinin, hemin, and antioxidants. (A) Ethanol alone; (B) artemisinin (1 mM); (C) artemisinin (1 mM) plus hemin (100 μM); (D) artemisinin (1 mM), hemin (100 μM), and deferoxamine (100 μM); (E) artemisinin (1 mM), hemin (100 μM), and α-tocopherol (100 μM). Hemin, deferoxamine, and α-tocopherol alone lowered thiol content by <10%.

on erythrocyte deformability (Fig. 4). When erythrocytes are incubated with either artemisinin (1 mM) or hemin (25 μM), there is no loss of deformability. However, when they are incubated with a combination of 25 μM hemin and 1 mM artemisinin, a marked decrease in the maximal deformability under isotonic conditions (around 290 mosM) is observed.

The preceding results are consistent with the hypothesis that the biological effects of artemisinin are mediated by an iron-catalyzed cleavage of the drug's endoperoxide group, generating free radicals. The capacity of this reaction to generate free radicals was then confirmed by EPR using the spin trap DMPO. In the absence of DMPO, neither artemisinin alone nor the combination of artemisinin plus iron generates any detectable signal (data not shown). However, a complex sextuplet with g values between 1.991 and 2.014 can be detected when artemisinin is incubated with iron in the presence of DMPO (Fig. 5A). In contrast, neither artemisinin (Fig. 5B) nor iron alone (Fig. 5C) produces a signal in the presence of DMPO.

DISCUSSION

In this paper, we show that the effects of artemisinin on both parasites and mice can be partially reversed by iron chelators, suggesting that iron plays an important role in the mechanisms of action and toxicity of this drug. We also show that both an iron chelator and a free radical scavenger antagonize artemisinin-induced oxidation of membrane protein thiols, suggesting that both iron and free radicals are involved in this process. Finally, we present EPR evidence that iron causes the generation of free radicals from artemisinin.

The apparent mechanism of action and toxicity of artemisinin is as follows:

$$\text{artemisinin} \xrightarrow{Fe} R\cdot \longrightarrow \text{free radical damage (lipid peroxidation, protein oxidation, alkylation, etc.)} \longrightarrow \text{cell death}$$

where R · is a free radical or mixture of free radicals.

The antagonistic effects of iron chelators on the activity and toxicity of artemisinin are consistent with several pre-

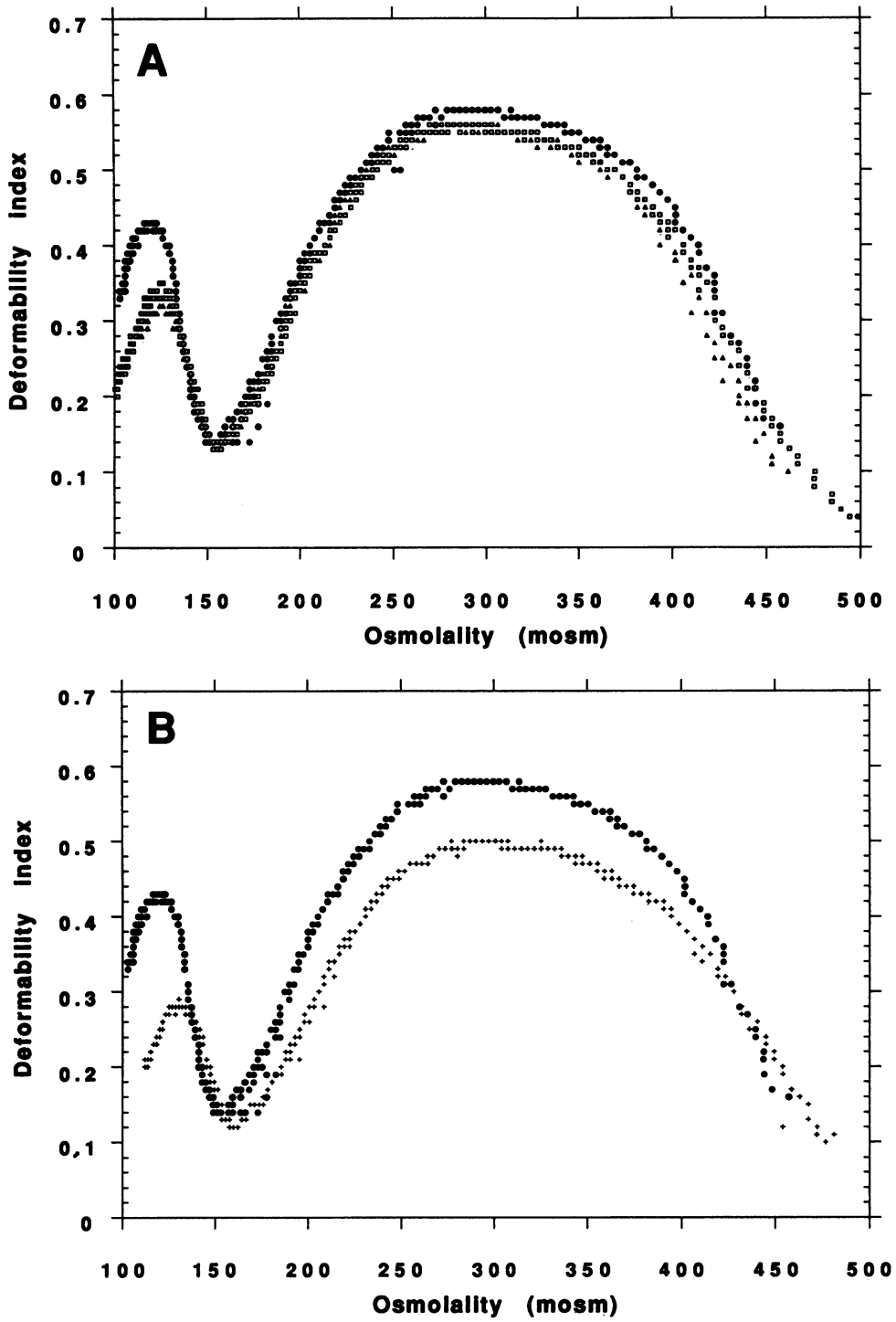

FIG. 4. Osmotic gradient deformability pattern of cells incubated for 30 min at 37°C in the absence (●) or presence of 25 μM hemin (▲) or 1 mM artemisinin (□) (A) and at the same temperature in the absence (●) or presence (+) of both 25 μM hemin and 1 mM artemisinin (B).

viously published observations. First, the prototypical iron chelator, DFO, has recently been demonstrated to antagonize the antimalarial activity of artemisinin and arteether (25). Second, heme iron has been shown to react with artemisinin (30) and to catalyze its electrochemical reduction (48). Third, iron has been shown to catalyze the decomposition of artemisinin into a reactive aldehyde (35). However, it is not clear why iron chelators are active if heme-bound iron is the catalyst. There are three possible explanations. First, iron chelators might bind directly to heme, as has been demonstrated for DFO (2, 44). Second, heme has been shown to be alkylated by artemisinin (30), and alkylated hemes tend to release free iron (29). This released iron might then be captured by the chelator. Finally, some of the

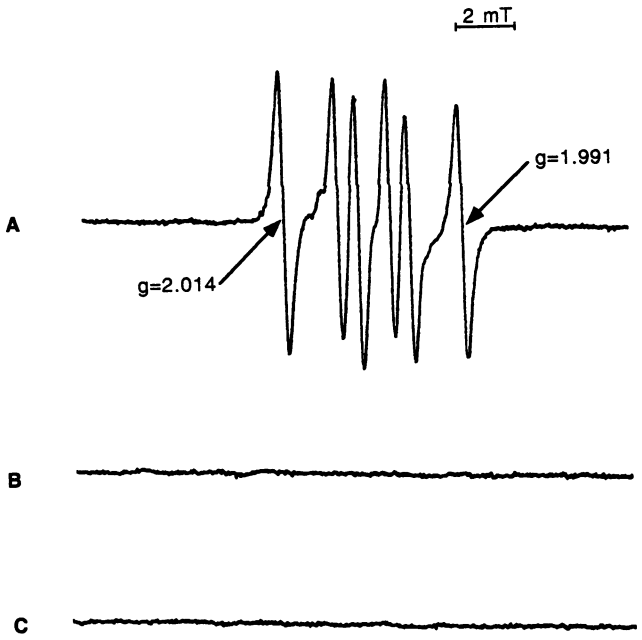

FIG. 5. EPR spectra of artemisinin in the presence and absence of iron and/or DMPO. (A) Artemisinin, iron and DMPO; (B) artemisinin and DMPO; (C) iron and DMPO.

important effects of artemisinin might, indeed, be due to nonheme iron.

This report contains the first direct evidence for the formation of a free radical from artemisinin and demonstrates that iron is required for a free radical to form. The EPR spectrum observed is characteristic of the oxidized DMPO that forms in the presence of hemin and various organic peroxides (14, 24). Earlier observations that artemisinin analogs lacking an endoperoxide moiety are inactive (6, 8) suggest that this reaction is essential for antimalarial activity. Other evidence for a free radical mechanism includes the observations that free radical scavengers antagonize the antimalarial effects of artemisinin (27, 28, 31) and that other oxidant drugs are synergistic with artemisinin (27). Additionally, various endpoints of free radical damage have been detected in artemisinin-treated erythrocytes and infected erythrocytes such as thiobarbituric acid-reacting substances (caused by lipid peroxidation) (31), methemoglobin formation (39), glutathione oxidation (39), and the oxidation of membrane protein thiols (30).

This mechanism explains why artemisinin is selectively toxic to malaria parasites. The human erythrocyte contains the equivalent of 20 mM heme, which is almost entirely bound to hemoglobin (20). Malaria parasites digest approximately 25% of the host hemoglobin (37) but do not catabolize heme (13). Instead, the accumulated heme is stored in food vacuoles as a polymer known as hemozoin (41). There are several lines of evidence that this heme iron then activates the drug. First, hemozoin from parasites exposed to [^{14}C]artemisinin concentrates the drug (30). Second, a chloroquine-resistant strain of the murine malaria parasite, *Plasmodium berghei*, which lacks hemozoin, is highly resistant to artemisinin (33). (In contrast, chloroquine-resistant *P. falciparum*, a human malaria parasite, contains hemozoin and is fully sensitive to artemisinin [32]). Finally, chloroquine, which binds heme avidly, is antagonistic towards artemisinin (7, 43), and chloroquine-mediated clumping of pigment granules is inhibited by artemisinin (33).

Iron chelators represent a potentially important new class of antimalarial agents. Several iron chelators have been shown to have antimalarial activity (see, for example, references 16, 21, 36, and 38), and some have already been used in patients (18, 45). The antimalarial activities of iron chelators appear to be dependent on their penetration into the parasite (15, 22, 40). However, our data suggest that iron chelators and artemisinin-class drugs might be less effective when used simultaneously.

DFO-HES is a new and nontoxic iron chelation agent which has shown promise as a potential therapy for iron overload (19). It does not penetrate the erythrocyte membrane and has little antimalarial activity (40). Chelator uptake may also be necessary for antagonism of artemisinin antimalarial activity. Thus, nonpermeating chelators such as DFO-HES might also be able to mitigate the toxicity of artemisinin derivatives without compromising their antimalarial effects. On the other hand, DMP, PBH, and related chelators, which antagonize arteether, pass through cell membranes (1, 23, 34).

Artemisinin is a remarkably nontoxic drug. Mice were not obviously affected by doses as high as 2 g/kg (data not shown). Since the therapeutic dose of artemisinin in mice is approximately 4 mg/kg (33), the drug clearly has a high therapeutic index. However, toxicity is always a potential problem; in monkeys, high doses have been shown to cause inhibition of hematopoiesis, as well as cardiac, renal, and hepatic toxicity (9). The most important toxicity in dogs and rats is a selective neurocytotoxicity in the central nervous system (5). The diminution of artemisinin's toxicity for mice by an iron chelator suggests that some of the toxicity of artemisinin to its host might be due to the same processes that are responsible for its toxicity to malaria parasites, namely, the iron-catalyzed generation of free radicals.

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