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Research Article

Plasmodium falciparum infecting hemoglobin (Hb) H and/or Hb Constant Spring erythrocytes in vitro was relatively more resistant than that infecting normal erythrocytes to artesunate and chloroquine, while the sensitivity to pyrimethamine was unchanged. The 50% inhibitory concentrations (IC50) for artesunate in HbH (alpha-thal 1/alpha-thal 2), HbH (alpha-thal 1/Hb Constant Spring), and homozygous Hb Constant Spring erythrocytes were 4.5 +/- 2.8, 8.5 +/- 3.2, and 2.6 +/- 1.6 nM compared with 0.82 +/- 0.35 nM in normal erythrocytes (P less than 0.002 for all three cases). The IC50 for chloroquine were 97 +/- 46, 162 +/- 67, and 93 +/- 36 nM, respectively, in the variant erythrocytes, compared with 48 +/- 13 nM in normal erythrocytes (P less than 0.002, 0.002, and 0.02, respectively). The differences in sensitivity to artesunate and chloroquine of the parasite infecting HbH erythrocytes are probably related to their oxidative mode of action and relatively high amounts of antioxidant enzymes in the host erythrocytes. This novel example of dependence on the host of the malarial parasite drug sensitivity may have implications for chemotherapy of malaria in patients with genetically variant erythrocytes.

Decreased Sensitivity to Artesunate and Chloroquine of *Plasmodium falciparum* Infecting Hemoglobin H and/or Hemoglobin Constant Spring Erythrocytes

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Abstract

Plasmodium falciparum **infecting hemoglobin (Hb) H and/or Hb Constant Spring erythrocytes in vitro was relatively more resistant than that infecting normal erythrocytes to artesunate and chloroquine, while the sensitivity to pyrimethamine was unchanged. The 50% inhibitory concentrations (IC_{50}) for artesunate in HbH (α-thal 1/α-thal 2), HbH (α-thal 1/Hb Constant Spring), and homozygous Hb Constant Spring erythrocytes were 4.5±2.8, 8.5±3.2, and 2.6±1.6 nM compared with 0.82±0.35 nM in normal erythrocytes ($P < 0.002$ for all three cases). The IC_{50} for chloroquine were 97±46, 162±67, and 93±36 nM, respectively, in the variant erythrocytes, compared with 48±13 nM in normal erythrocytes ($P < 0.002$, 0.002, and 0.02, respectively). The differences in sensitivity to artesunate and chloroquine of the parasite infecting HbH erythrocytes are probably related to their oxidative mode of action and relatively high amounts of antioxidant enzymes in the host erythrocytes. This novel example of dependence on the host of the malarial parasite drug sensitivity may have implications for chemotherapy of malaria in patients with genetically variant erythrocytes.**

Introduction

Malaria caused by *Plasmodium falciparum* is the most frequently occurring form throughout the tropics and subtropics (1). In these regions the frequencies of hemoglobinopathies are also high, and it has been shown by gene mapping (2, 3) that natural selection by malaria is probably the factor responsible for high frequencies of α-thalassemia. Impaired growth of *P. falciparum* in vitro has been demonstrated in hemoglobin (Hb) H erythrocytes from persons with α-thalassemia (α-thal 1/α-thal 2 or $--/-\alpha$ genotype) (4, 5). Impaired growth was also found in another type of HbH erythrocytes from persons with α-thalassemia in combination with Hb Constant Spring (CS)[1] (α-thal 1/HbCS or $--/\alpha^{cs}\alpha$ genotype) and in homozygous HbCS erythrocytes (5). In addition, the infected variant erythrocytes showed an increased susceptibility to phagocytosis by monocytes (5). These results indicate the important role of variations in the α-globin gene in parasite–host interaction. However, the problem of parasite–host–drug interaction has been little investigated, especially with regard to α-globin gene variations. Since the frequencies of α-thalassemia in southeast Asia, for example, are as high as 20–30% and the frequencies of HbCS up to 8% (6), this problem assumes importance because of possible implications in treatment of infected variant individuals and in origins of drug resistance in the endemic areas.

Among the various consequences of α-globin gene variations, increased oxidant stress is potentially important (7). Increased amounts of antioxidant enzymes found for HbH (α-thal 1/α-thal 2) and HbH/CS (α-thal 1/HbCS) erythrocytes (8, 9) are probably due to compensation mechanisms against the increased oxidant stress. Since *P. falciparum* is sensitive to oxidant stress (10–12) and oxidant drugs (13), its interaction with the variant erythrocytes and oxidant drugs, especially those developed for widespread use, deserves detailed investigation. Artesunate is a derivative of artemisinin (qinghaosu), an antimalarial natural product originally developed in China (14). The endoperoxide group is essential for their activities (14), and we have recently shown (15) that there is potentiation between these drugs and oxidant drugs or oxygen, and that reducing agents decrease their effectiveness against *P. falciparum* in vitro. These drugs, therefore, probably act through an oxidative mode of action. This report shows that *P. falciparum* sensitivity to artesunate is reduced in infection of variant erythrocytes carrying HbH with or without HbCS, and homozygous HbCS erythrocytes, in contrast with unchanged sensitivity to pyrimethamine which acts through a nonoxidative mode.

Methods

Materials. Artesunate was a gift from the Chinese authorities through the World Health Organization. Chloroquine diphosphate was from Sigma Chemical Co., St. Louis, MO. Pyrimethamine was a gift from Walter Reed Army Institute of Research, Washington, D.C. Genetically variant blood samples were typed and supplied by the Hematology Unit, Siriraj Hospital, Bangkok.

Methods. Chloroquine-resistant *P. falciparum,* K1 strain, was grown in type O erythrocytes by the candle jar method of Trager and Jensen (16). Synchronous cultures were obtained by treatment with 5% sorbitol (17). Concentrated schizont-infected erythrocytes (~ 98% parasitemia) were obtained from a Percoll layering method (18) and subcultured in genetically variant or normal erythrocytes at an initial parasitemia of 0.2–0.3%. The antimalarial activity of the drugs was tested by the modified method of Rieckmann et al. (19). Medium was changed at 24 h, and at 36 h the cultures were exposed as 3% cell suspensions in 24-well plastic plates to various concentrations of drugs for another 96 h with a change in drug-containing media every 24 h. Artesunate-containing media were prepared by adding the drug in DMSO solution to a final concentration of 0.1% DMSO, which had no

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1. *Abbreviations used in this paper:* CS, Constant Spring; G6PD, glucose-6-phosphate dehydrogenase; IC_{50}, 50% inhibitory concentration.

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effect on parasite development and multiplication. Parasitemia was determined by counting the number of parasites per 10,000 erythrocytes in Giemsa-stained thin blood films.

The concentrations of drugs that inhibited parasite multiplication by 50% (IC_{50}) were determined from the effect of six drug concentrations obtained from threefold serial dilutions. The inhibition was estimated by comparisons with controls in which the parasites were cultured with the variant erythrocytes in the absence of drugs. Data were analyzed using the Mann-Whitney U test for comparing two populations based on independent random samples.

Results

Higher concentrations of artesunate were required to kill *P. falciparum* infecting the genetically variant erythrocytes in vitro than that infecting normal erythrocytes. The IC_{50} values for artesunate against the parasite infecting normal, HbH (α-thal 1/α-thal 2), HbH/CS (α-thal 1/HbCS), homozygous HbCS and heterozygous HbCS erythrocytes are shown in Fig. 1. These values (mean±SD) are, respectively, 0.82±0.35 nM, 4.5±2.8 nM ($P < 0.002$), 8.5±3.2 nM ($P < 0.002$), 2.6±1.6 nM ($P < 0.002$), and 1.29±0.17 nM ($P < 0.05$). High concentrations were also required for chloroquine in infection in variant erythrocytes except in heterozygous HbCS erythrocytes, although the magnitudes of the increase are smaller (Fig. 2). The IC_{50} values are 48±13 nM, 97±46 nM ($P < 0.002$), 162±67 nM ($P < 0.002$), 93±36 nM ($P < 0.02$), and 44±8 nM (NS) for HbH, HbH/CS, homozygous HbCS, and heterozygous HbCS erythrocytes, respectively.

Similar to our previous report (5), the multiplication ratio, i.e., the ratio of % parasitemia at 96 h compared with that at 0 h, in the absence of drugs is slightly but significantly higher for normal erythrocytes (32.4±9.6) than for HbH (24.6±9.2, $P < 0.02$), HbH/CS (21.1±6.4, $P < 0.02$), homozygous HbCS (26.0±8.8, $P < 0.02$), and heterozygous HbCS (20.4±1.5, $P < 0.02$). The possibility that these intrinsic differences in multiplication might be related to the differences in IC_{50} observed with artesunate and chloroquine was ruled out by a control experiment with pyrimethamine. In contrast with artesunate and chloroquine, the effectiveness of pyrimethamine against the parasite infecting the variant erythrocytes was the same as against that infecting normal erythrocytes (Fig. 3). The IC_{50} values are 16.6±3.9 nM, 15.7±4.6 nM (NS), 16.9±2.6 nM (NS), 16.7±4.6 nM (NS), and 14.3±0.5 nM (NS) for normal, HbH, HbH/CS, homozygous HbCS, and heterozygous HbCS erythrocytes, respectively.

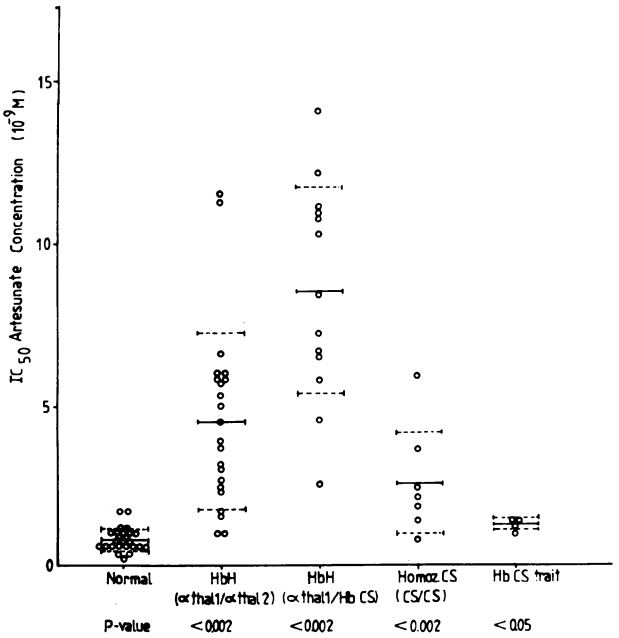

Figure 1. The IC_{50} values with means±SD for artesunate against *P. falciparum* infecting normal, HbH (α-thal 1/α-thal 2), HbH/CS (α-thal 1/HbCS), homozygous HbCS, and HbCS trait erythrocytes.

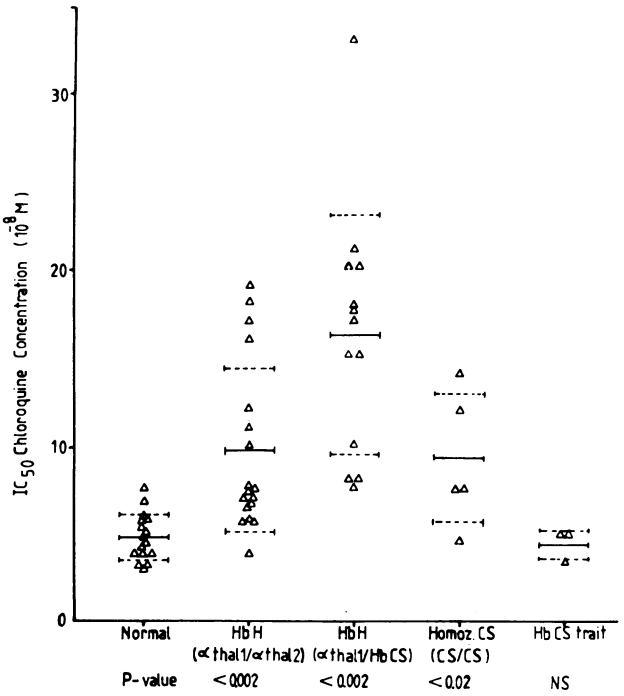

Figure 2. The IC_{50} values with means±SD for chloroquine against *P. falciparum* infecting normal, HbH (α-thal 1/α-thal 2), HbH/CS (α-thal 1/HbCS), homozygous HbCS, and HbCS trait erythrocytes.

Discussion

Although host genetic factors are considered important in antimalarial drug testing (20), major concern up to now has been

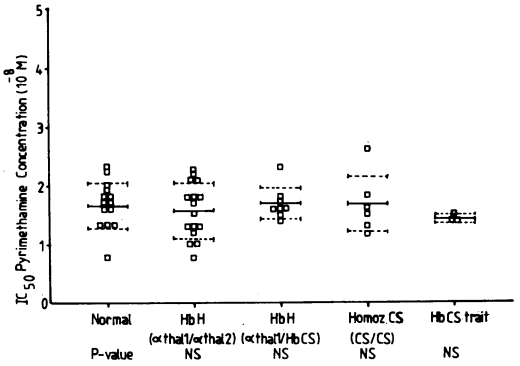

Figure 3. The IC_{50} values with means±SD for pyrimethamine against *P. falciparum* infecting normal, HbH (α-thal 1/α-thal 2), HbH/CS (α-thal 1/HbCS), homozygous HbCS, and HbCS trait erythrocytes.

placed on the toxic effects of the antimalarials on the host or on host drug metabolism. There has been no previous study of the effect of antimalarials on the parasite growing in variant erythrocytes. Our results show that the antimalarials artesunate and chloroquine had less activity against *P. falciparum* growing in α-thalassemia and/or HbCS erythrocytes than normal erythrocytes (Figs. 1 and 2), while the activity of pyrimethamine was the same for all erythrocytes. Among possible factors that can account for this observation is the sensitivity to oxidative damage of *P. falciparum* infecting the variant erythrocytes (10). Friedman (10) has shown that *P. falciparum* in glucose-6-phosphate dehydrogenase (G6PD)–deficient, α- and β-thalassemic erythrocytes are more sensitive than normal to conditions of increased oxidant stress. Further preliminary evidence was found (21) to suggest that some metabolite of the fava bean may be inhibitory to *P. falciparum* growing in β-thalassemia trait or G6PD-deficient erythrocytes, but not to normal erythrocytes. Isouramil, a fava bean extract known to cause oxidant stress, was found to inhibit the growth of the parasite in G6PD-deficient erythrocytes (22). These results suggest that the oxidant sensitivity of *P. falciparum* may play a role in the protective effect of variant erythrocytes.

Artesunate, which depends for its antimalarial activity on its endoperoxide group (14), probably acts through an oxidative mode. Evidence for this includes potentiation with oxidant drugs or oxygen and reduced effectiveness in the presence of reducing agents (15). It might be expected, therefore, that the variant erythrocytes studied would confer increased sensitivity to artesunate on the parasite. The fact that opposite results were observed can be explained on the basis that the drug's oxidative action is different from that generated by oxygen or other nonantimalarial oxidants, and that the variant erythrocytes are relatively protected from induced drug oxidative damage. Artesunate and artemisinin exert a predominant effect specifically on parasite membrane systems (23, 24), while other nonantimalarial oxidative agents exert only nonspecific damage on the host–parasite complex. In addition, the levels of antioxidant enzymes glutathione peroxidase and superoxide dismutase in HbH and HbH/HbCS erythrocytes are significantly higher than normal erythrocytes (8, 9) and may help reduce more effectively oxidant stress generated by the drug.

It was recently shown (25) that the sensitivity of *Plasmodium berghei* to artemether, another qinghaosu derivative, was different for the parasites infecting phenylhydrazine-treated mice and irradiated mice, the ED_{50} for the former being about three times lower than the latter. This was interpreted to be due to the lower host cell age of the phenylhydrazine-treated mice. The reduced lifespan of homozygous HbCS erythrocytes (26) suggests that host cell age may be a contributing factor to the observed differences in drug sensitivities. On the other hand, phenylhydrazine treatment may produce, in addition to reticulocytosis, other host cell changes similar to conditions prevailing in the genetically variant erythrocytes.

The different sensitivities of *P. falciparum* in genetically variant erythrocytes to chloroquine are difficult to explain since the mechanism of chloroquine action is presently unknown. The data presented in this report suggest that the action might involve enhancement of oxidant stress, although more definitive results are required. Another possibility is reduced uptake of the drug by infected variant erythrocytes. In contrast, the sensitivity of *P. falciparum* to pyrimethamine was found to be independent of the host erythrocytes, a result expected because the drug acts on the folate biosynthetic pathway which should not be subject to direct interference by modulation of oxidant stress.

Regardless of the mechanisms of action of artesunate and chloroquine, the results reported here should have implications for the chemotherapy of falciparum malaria in genetically variant individuals. In view of the high frequencies of α-thalassemia and HbCS in various endemic regions, the host dependence of drug sensitivities may also have significant epidemiological implications. This problem assumes enhanced significance considering the early emergence of chloroquine resistance in southeast Asia (27) and the high recrudescence rate of falciparum malaria patients treated with qinghaosu (28).

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