Hemolysis of Mouse Erythrocytes by Ferriprotoporphyrin IX and Chloroquine

CHEMOTHERAPEUTIC IMPLICATIONS

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ABSTRACT Incubation of a 0.5% suspension of washed normal mouse erythrocytes with ferriprotoporphyrin IX (FP) for 2.5 h at 37°C and pH 7.4 results in sufficient membrane damage to produce hemolysis. A sigmoidal dose-response curve is followed with 50% hemolysis being produced by 4 μM FP. Complete hemolysis is produced by 6 μM FP. The hemolytic process has at least two phases: a lag phase of ~45 min, during which little hemolysis occurs, and a phase characterized by rapid hemolysis. Chloroquine, which binds tightly to FP, enhances the effect of FP by eliminating the lag phase. Under the conditions of these experiments, maximum enhancement is observed with chloroquine concentrations in the range of 5–25 μM. Since FP is produced when malaria parasites digest hemoglobin, it may mediate a chemotherapeutic effect of chloroquine by forming a complex with the drug that could enhance the toxicity of FP for biological membranes, including those of the parasite.

INTRODUCTION

Ferriprotoporphyrin IX (FP)[1] is produced when intraerythrocytic malaria parasites digest hemoglobin. Although this compound is clearly a waste product of the parasites' metabolism, it fulfills certain criteria for identification as an important drug receptor. It has high affinity (K_{diss} = 3.5 nM) and the appropriate specificity for chloroquine and related 4-aminoquinoline drugs (1). In the role of a drug receptor, FP could mediate the chemotherapeutic action of chloroquine in either of two ways. It could serve only to concentrate the drug in the proper location, or the FP-chloroquine complex could itself be toxic to the malaria parasite. We now demonstrate the plausibility of the latter possibility by showing that chloroquine accelerates FP-induced hemolysis.

METHODS

Normal mouse erythrocytes were used as a model system, and hemolysis was used as an indicator of membrane damage. The methods for preparing washed erythrocytes and the standard medium used in this work have been described (1, 2). This medium is buffered to pH 7.4 with 50 mM phosphate. Chloroquine diphosphate and FP (hemin from equine blood) were purchased from Sigma Chemical Co. St. Louis, Mo. The FP was recrystallized once according to the procedure of Labbe and Nishida (3). On the day of each experiment, a fresh stock solution of 0.5 mM FP was prepared in 5 mM NaOH and placed in an ice bath. Immediately before use, an aliquot of the stock solution was diluted to the appropriate concentration using ice-cold standard medium, pH 7.4. The erythrocytes were incubated with FP and chloroquine in plastic vessels in an Eberbach shaking incubator operating at 2.3 Hz. Hemolysis was measured as follows. At the end of incubation, intact erythrocytes were sedimented by centrifugation, and the amount of hemoglobin in the supernatant solution was determined by measuring the OD at 540 nm. Then the erythrocyte pellet was lysed with distilled water, and its hemoglobin content was measured. Percent hemolysis was calculated from these measurements.

RESULTS

The hemolysis of erythrocytes by FP followed a sigmoidal dose-response curve (Fig. 1). Under the conditions of the experiment, 50% hemolysis was produced by 4 μM FP. This amount of FP is approximately equal to 4% of the heme in the erythrocyte suspension. The hemolytic process induced by FP has at least two phases (Fig. 2). There was a lag phase of ~45 min, during which time little hemolysis occurred. Then, there was a phase characterized by rapid hemolysis. The lag phase could be prolonged by decreasing the incubation temperature and shortened by increasing the concentration of FP. Conducting the incubation in the dark did not diminish the hemolysis,

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[1] *Abbreviation used in this paper*: FP, ferriprotoporphyrin IX.

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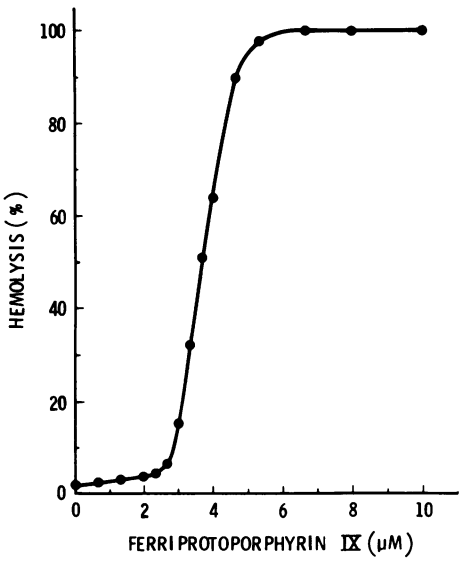

FIGURE 1 Hemolytic effect of FP. Aliquots of a 0.5% suspension of thrice-washed normal mouse erythrocytes were incubated with various concentrations of FP for 2.5 h under room air at 37°C and pH 7.4. In the absence of FP, <3% hemolysis was observed.

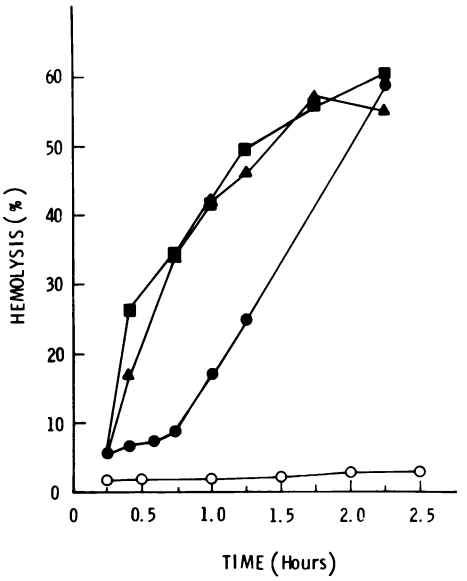

FIGURE 2 Effect of chloroquine on FP-induced hemolysis. Aliquots of a 0.5% suspension of thrice-washed normal mouse erythrocytes were preincubated for 15 min under room air at 37°C and pH 7.4 either in the presence or absence of 5 μM FP. Then chloroquine was added and the incubation was continued. FP alone (●), FP plus 2.5 μM chloroquine (▲), FP plus 5 μM chloroquine (■), no addition of FP or chloroquine (○). In the absence of FP, chloroquine caused no hemolysis.

indicating that protoporphyrin IX-induced photohemolysis is not involved.

Chloroquine enhanced the toxicity of FP by accelerating the hemolytic process (Fig. 2). In fact, rapid hemolysis began immediately upon the addition of chloroquine. In Fig. 2, the chloroquine was added after the erythrocytes had been incubated with FP for 15 min, but the results were the same when FP and chloroquine were added together at the beginning of the incubation. The concentration dependence of the effect of chloroquine is shown in Fig. 3. A bell-shaped curve was produced with the peak effect occurring between 5 and 25 μM chloroquine. Therapeutic concentrations of chloroquine in whole blood may be as high as 5 μM (4), but much lower concentrations are usually effective in treating malaria (5). Since our experiments used only relatively short incubation periods and a gross indicator of membrane damage, it is possible that exposure to low concentrations of the FP-chloroquine complex in vivo would be sufficient to produce subtler but nonetheless lethal damage. The reason for less effect of high concentrations of chloroquine in the hemolytic test shown in Fig. 3 has not been determined; possibly it is related to the extent of saturation of FP by the drug.

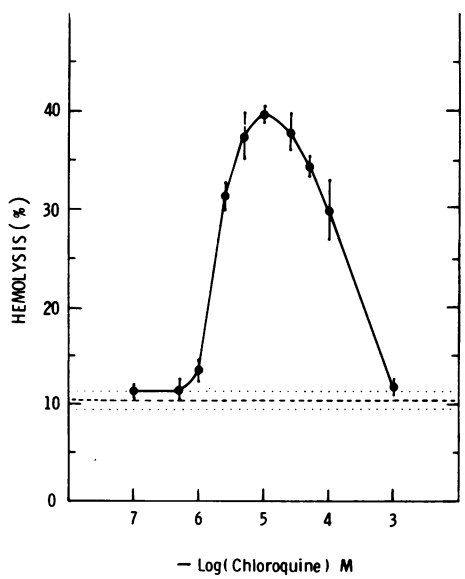

FIGURE 3 Concentration dependence of the effect of chloroquine on FP-induced hemolysis. Aliquots of a 0.5% suspension of thrice-washed normal erythrocytes were preincubated with 5 μM FP as in Fig. 2 before incubation with various concentrations of chloroquine for 30 min. Means and SE are shown for three separate experiments. The dotted lines show the results of control incubations with FP only.

DISCUSSION

Since FP is lytic for the erythrocyte membrane, it is reasonable to suspect that it would damage various other biological membranes, including those of the

malaria parasite. In the parasite, FP accumulates in malarial pigment in digestive vacuoles (6–8). Consequently, the membranes surrounding these vacuoles should be the most vulnerable. Sequestration of FP in malarial pigment (8) may ordinarily keep its concentration very low. The presence of chloroquine, however, might enhance the toxicity of low concentrations of FP sufficiently to cause membrane damage. Such damage to the digestive vacuoles could cause them to fuse and to form the large vesicles which are known to be the first morphologic manifestation of the chemotherapeutic action of chloroquine (9, 10). Therefore, it is plausible to propose that FP not only serves to recognize chloroquine but, in addition, mediates a chemotherapeutic effect of this drug on biological membranes.

It is also noteworthy that FP is generated in hemolytic anemias associated with the production of Heinz bodies (11, 12) and possibly could contribute to hemolysis in some of them.

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