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SCIENCE

Hemin Lyses Malaria Parasites

Abstract. *Malaria parasites isolated from mouse erythrocytes are lysed by ferriprotoporphyrin IX chloride (hemin) or by a chloroquine-hemin complex in amounts that could be produced by release of less than 0.1 percent of the heme in erythrocytic hemoglobin. This effect of hemin may explain the protection against malaria provided by thalassemia and other conditions causing intracellular denaturation of hemoglobin. The toxicity of the chloroquine-hemin complex may explain the selective antimalarial action of chloroquine.*

Recently it was found that ferriprotoporphyrin IX chloride (hemin) lyses *Trypanosoma brucei* (*1*) and normal erythrocytes (*2*) and that the formation of a chloroquine-hemin complex in erythrocytes does not inhibit hemolysis (*2*). These observations have important implications for the biology and chemotherapy of malaria. Erythrocytic malaria parasites exist in an environment rich in hemoglobin and could be exposed to toxic amounts of heme when hemoglobin undergoes denaturation, as occurs spontaneously in hemolytic anemias associated with the production of Heinz bodies (*3*). Malaria parasites also degrade hemoglobin and produce a large amount of heme, which normally is sequestered in malaria pigment (*4*), presumably in an innocuous form. On exposure to chloroquine, however, erythrocytes infected with malaria parasites accumulate a chloroquine-heme complex (*5*) that could be toxic. In this report we provide evidence that hemin and a chloroquine-hemin complex are indeed toxic to malaria parasites.

The NYU-2 strain of *Plasmodium berghei* was studied. Two lines (*6*) of this strain, one chloroquine-susceptible and the other chloroquine-resistant, are available in our laboratory. The former produces abundant pigment and the latter no detectable pigment in mouse erythrocytes. To minimize confounding of the data by the parasites' own heme, the chloroquine-resistant line was chosen for the present study. (Preliminary experiments with the chloroquine-susceptible line have shown that it responds to hemin and the chloroquine-hemin complex in the same way as the chloroquine-resistant line.)

Parasites were obtained from the blood of heavily infected male Swiss-Webster mice (parasitemia in excess of 30 percent). The blood from several mice was pooled and mixed with an equal volume of a standard isotonic medium (*p*H 7.4) (*7*) containing 1 mg of heparin per milliliter. White blood cells were removed by passing this mixture through a column of Whatman CF 11 cellulose powder. The erythrocytes were then recovered by centrifugation, suspended in 10 volumes of standard medium, and centrifuged again. To lyse the erythrocytes, the washed pellet was suspended in 20 volumes of 0.015 percent (weight to volume) saponin (Sigma) in the standard medium and incubated at 37°C for 30 minutes. Parasites were isolated from the lysate by centrifugation and washed three times by suspension in 40 volumes of cold standard medium and centrifugation at 4°C. All centrifugations were conducted at 500*g* for 15 minutes.

Figure 1 shows photomicrographs of

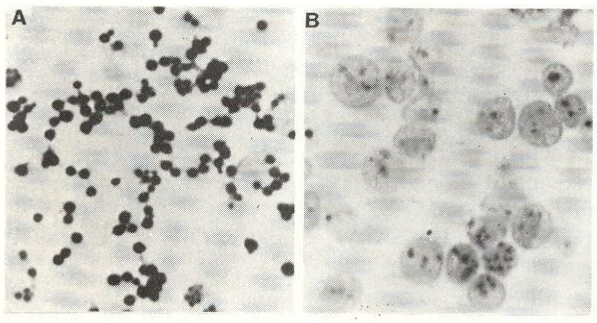

Fig. 1. Microscopic appearance of isolated *P. berghei* (×1100). (A) Control view. The parasites were suspended (10^7 per milliliter) in the standard medium and incubated. At the end of the incubation period glutaraldehyde was added to achieve a final concentration of 2 percent and the mixture was left at room temperature for 20 minutes. The mixture was centrifuged and the pellet was washed once with standard medium. Smears of the washed pellet were made on albumin-coated glass slides, air-dried, and stained. (B) Parasites from a portion of the same suspension used in (A), incubated in the presence of 20 μ*M* hemin. Treatment of the parasites with a complex formed from 20 μ*M* hemin and 5 μ*M* chloroquine produced results similar to those seen in (B); chloroquine alone caused no change.

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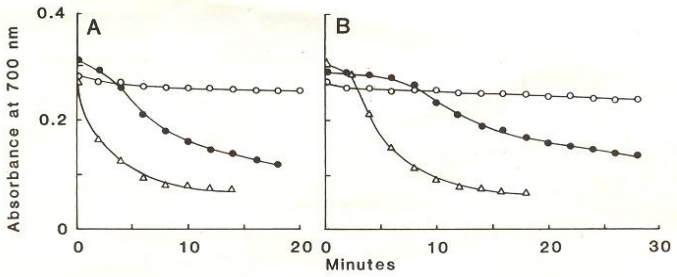

Fig. 2. Time course of the effect of hemin on the turbidity of *P. berghei* suspensions. Isolated parasites were suspended (10^7 per milliliter) in the standard medium and incubated at 37°C with no hemin (○) or with 10 μ*M* (●) or 20 μ*M* (Δ) hemin. (A) The results obtained when no chloroquine was present in the incubation medium; (B) the results when 5μ*M* chloroquine was present. In the experiments in which both hemin and chloroquine were present, the two compounds were mixed before being added to the suspension of parasites. Approximately 30 seconds elapsed between the addition of hemin or the chloroquine-hemin complex and the first measurement of absorbance shown in the figure. The absorbance at 700 nm was measured with a Beckman model 25 spectrophotometer.

the isolated parasites, fixed with glutaraldehyde and stained with Giemsa. There was minimal contamination by intact erythrocytes. In the absence of hemin the parasites appeared as small blue cells with barely visible red nuclei; at 2°C, they retained their normal appearance for several hours (Fig. 1A). After exposure to hemin from equine blood (Sigma) or the chloroquine-hemin complex for 10 minutes, the parasites became swollen, and there was a decrease in cytoplasmic staining (Fig. 1B). The swelling produced by hemin (20 μ*M* or higher) was maximal within 10 minutes (*8*).

The time course of swelling was evaluated by measuring the turbidity of parasite suspensions. On exposure to hemin there was a rapid decrease in absorbance at 700 nm (Fig. 2). Concomitant with the swelling, glutamic acid dehydrogenase (E.C. 1.4.1.4) was released from the parasites (Fig. 3) (*9*), indicating loss of intracellular contents. These findings point to lysis of the parasites.

Addition of chloroquine diphosphate (Sigma) to hemin to form a complex delayed but did not prevent lysis (Fig. 2). Only the effect of 5 μ*M* chloroquine is shown in Fig. 2, but the responses to 1 and 10 μ*M* chloroquine were also studied. The delay was less with 1 μ*M* chloroquine and somewhat greater with 10 μ*M* chloroquine. In equilibrium dialysis experiments, it has been found that a maximum of one molecule of chloroquine can be bound by two molecules of hemin (*5*). In agreement with the erythrocyte model of Chou and Fitch (*10*), chloroquine alone did not cause malaria parasites to swell or lyse.

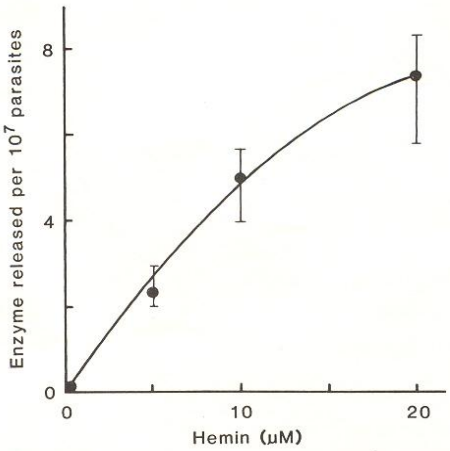

Fig. 3. Release of glutamic acid dehydrogenase from *P. berghei* in the presence of hemin. Isolated parasites were suspended (10^7 per milliliter) in the standard medium and incubated with the desired concentration of hemin at 37°C for 10 minutes. The parasites were then removed by centrifugation (3000*g* for 5 minutes at 4°C) and the supernatant solution was used for measurement of glutamic acid dehydrogenase activity. The ordinate shows enzyme activity expressed as nanomoles of α-ketoglutarate per minute. The means and ranges for three experiments are shown. In control studies without hemin, which were included in each experiment, no enzyme release was detectable.

Considering that the hemoglobin concentration in erythrocytes is approximately 34 g per 100 ml of packed cells and that the molecular weight of hemoglobin is approximately 64,000, release of less than 0.1 percent of the heme in erythrocytic hemoglobin would be sufficient to produce intracellular heme concentrations as high as 20 μ*M*. The amount of heme released when hemoglobin undergoes denaturation intracellularly in such conditions as thalassemia and sickle cell anemia has not been measured, although denaturation is known to accelerate heme release (*3*). Since the release of only a small proportion of erythrocytic heme would be enough to lyse malaria parasites, however, heme toxicity may explain the protection against malaria provided by thalassemia and other conditions associated with intracellular denaturation of hemoglobin.

Chloroquine-susceptible malaria parasites may also be exposed to heme from within, since they degrade hemoglobin and sequester large amounts of heme in the form of malaria pigment (*4*). This sequestered heme is not available to bind chloroquine (*5*) and apparently causes little or no toxicity. By contrast, when susceptible parasites are exposed to chloroquine, heme accumulates in a different form that is available to bind the drug (*5*). The biochemical explanation for accumulation of heme as a drug complex rather than as malaria pigment remains unknown. Nevertheless, erythrocytes infected with the chloroquine-susceptible line of *P. berghei* accumulate 20 μmole of chloroquine per kilogram or more as the chloroquine-heme complex (*5*, *6*). Since the present results demonstrate that this amount is more than enough to lyse isolated malaria parasites, we propose that the selective antimalarial action of chloroquine is due to the accumulation of a toxic chloroquine-heme complex. Although the mechanism underlying this toxicity has not yet been studied, it may be similar to that observed in the erythrocyte model (*10*).

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References and Notes

1. S. R. Meshnick, K.-P. Chang, A. Cerami, *Biochem. Pharmacol.* **26**, 1923 (1977).
2. A. C. Chou and C. D. Fitch, *J. Clin. Invest.* **66**, 856 (1980).
3. H. S. Jacob and K. H. Winterhalter, *Proc. Natl. Acad. Sci. U.S.A.* **65**, 697 (1970); *J. Clin. Invest.* **49**, 2008 (1970); T. G. Gabuzda, in *Drugs and Hematologic Reactions*, N. V. Dimitrov and J. H. Nodine, Eds. (Grune & Stratton, New York, 1974), p. 49.
4. J. D. Fulton and C. Rimington, *J. Gen. Microbiol.* **8**, 157 (1953); K. A. Yamada and I. W. Sherman, *Exp. Parasitol.* **48**, 61 (1979).
5. A. C. Chou, R. Chevli, C. D. Fitch, *Biochemistry* **19**, 1543 (1980); C. D. Fitch and R. Chevli, *Antimicrob. Agents Chemother.* **19**, 589 (1981).
6. C. D. Fitch, N. G. Yunis, R. Chevli, Y. Gonzalez, *J. Clin. Invest.* **54**, 24 (1974); C. D. Fitch, R. Chevli, Y. Gonzalez, *J. Pharmacol. Exp. Ther.* **195**, 389 (1975).
7. C. D. Fitch, R. Chevli, Y. Gonzalez, *Antimicrob. Agents Chemother.* **6**, 757 (1974).
8. A stock solution of 1 m*M* hemin was prepared freshly on the day of an experiment by dissolving 16.3 mg of hemin in 25 ml of 0.02*N* NaOH and kept on ice until final dilutions were made with the standard medium. After dilution the *p*H of solutions containing hemin was 7.4
9. The method of B. W. Langer, Jr., P. Phisphumvidhi, and D. Jiampermpoon [*Exp. Parasitol.* **28**, 298 (1970)] was used to measure glutamic and dehydrogenase activity.
10. A. C. Chou and C. D. Fitch, *J. Clin. Invest.* **68**, 672 (1981). In the erythrocyte model, exposure to hemin impairs the ability of the cell membrane to maintain cation gradients, and there is massive loss of potassium, the cell swells, and lysis eventually occurs.
11. This work was supported by the United Nations/World Bank/World Health Organization Special Programme for Research and Training in Tropical Diseases.

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