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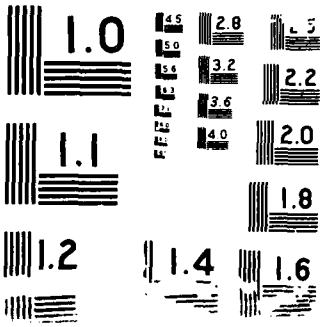
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DRUG RESISTANCE IN MALARIA: INVESTIGATION OF MECHANISMS AND PATTERNS OF DRUG RESISTANCE AND CROSS RESISTANCE IN MALARIA

ANNUAL/FINAL REPORT

COY D. FITCH, M.D.

October 19, 1987

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19. ABSTRACT (Continue on reverse if necessary and identify by block number)

This contract had five technical objectives: 1) identification of binding sites of drugs (mefloquine, etc.) in erythrocytes and/or parasites, 2) investigation of binding of drugs (mefloquine in particular) in malaria strains resistant and susceptible to other drugs (chloroquine in particular), 3) investigation of the mechanism of action of drugs (mefloquine in particular) and the effect on metabolism of erythrocytes and parasites, 4) correlation of drug resistance and accumulation of drugs by infected erythrocytes to allow predictions of cross resistance, and 5) development of a method to predict cross resistance based on response of isolated malaria parasites to drug-receptor complexes. As the experimental work progressed, if became apparent that most of our effort should be focused on the third objective.
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Block 19 (continued)

The experiments performed under this contract were guided by the hypothesis that "chloroquine acts by delaying the sequestration of ferriprotoporphyrin IX (FP) into malaria pigment, thereby allowing FP to exert its intrinsic cellular toxicity." Thus, the lytic effect of FP on biological membranes was studied in detail, using the human erythrocyte as the experimental cell. This work led to the proposal that FP is a lytic agent because it binds to membrane phospholipids and causes sufficient structural perturbation to disrupt the membrane. Furthermore, we obtained evidence that FP is released when hemoglobin is denatured in sickle cells and in G6PD-deficient erythrocytes and may be involved in the premature lysis of these cells in vivo and in their ability to protect against malaria.

The second line of investigation sought to determine how malaria detoxifies FP and makes it inaccessible to chloroquine and other antimalarial drugs. Three significant observations resulted from this line of investigation: 1) FP is sequestered in malaria pigment as a peculiarly insoluble, low oxygen form of hematin, 2) hydrogen peroxide in the parasitized erythrocyte may detoxify FP, and 3) chloroquine greatly delays the decomposition of FP by hydrogen peroxide. Consequently, chloroquine may cause the accumulation of a toxic chloroquine-FP complex either by delaying the sequestration of FP in malaria pigment or by delaying the decomposition of FP by hydrogen peroxide and perhaps other oxidants.

FOREWORD

Drs. Augustine U. Orjih, Stephen K. Janney, J. Heinrich Joist, and Jozef S. Murk, and Mrs. Rekha Chevli and Mrs. Phitsamai Kanjananggulpan participated in this work, and they are coauthors of published manuscripts describing the work. In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985).

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STATEMENT OF THE PROBLEM

One of the common malaria parasites of humans, Plasmodium falciparum, is capable of developing resistance to chloroquine. At present, chloroquine resistant strains of P. falciparum are prevalent in Southeast Asia and in Central and South America, and they are beginning to appear in Africa and other parts of the world. Because American soldiers are especially vulnerable when exposed to malaria and because the drugs available to effectively treat chloroquine-resistant malaria are so few, the United States Army has an interest in the development of new antimalarial drugs. In an effort to improve our armamentarium against malaria, a broad based effort is being made by the scientific community, which includes studies of the basic biology of the malaria parasite and vaccine development as well as drug development.

Our hypothesis to explain the mode of action of chloroquine is that chloroquine acts by delaying the sequestration of ferriprotoporphyrin IX (FP) into malaria pigment, thereby allowing FP to exert its intrinsic cellular toxicity. Therefore, the experimental work supported by this contract became focused on understanding the biochemical basis of FP toxicity and on an elucidation of the process of FP detoxification.

BACKGROUND

Evidence that chloroquine-susceptible malaria parasites possess a high-affinity drug receptor for chloroquine was published in 1969 for Plasmodium berghei (1) and in 1970 for a malaria parasite of humans, P. falciparum (2). By 1974, the topology of this receptor had been deduced from specificity studies (3), and attempts to isolate and characterize it became feasible. Six years later (4) the chloroquine receptor of malaria parasites was identified as ferriprotoporphyrin IX (FP). Since then rapid progress has been made. In 1981, chloroquine was shown to trap FP in the form of a drug receptor complex in malaria parasites by demonstrating that an exogenous chloroquine-FP complex behaved identically to the endogenous drug-receptor complex in cell-free preparations of malaria parasites (5). Also in 1981, FP and the chloroquine-FP complex were found to lyse P. berghei (6). This observation was confirmed for P. falciparum in 1982 (7). At about the same time it was discovered that the toxicity of FP for P. berghei can be eliminated by binding it either to exogenous or to parasite heme-binding-substances (8). From this series of observations we hypothesized that chloroquine acts as an antimalarial drug by diverting FP into a toxic chloroquine-FP complex. In the absence of chloroquine, FP would be sequestered in a nontoxic form in the malaria parasite. This hypothesis synthesized all of the available information about chloroquine susceptibility in malaria parasites, including the absolute requirement for hemoglobin digestion (9), and explained the selectivity of chloroquine as an antimalarial drug.

The mechanism of the toxicity of FP and the chloroquine-FP complex for biological membranes has received some attention but is still incompletely understood. In 1980 and 1981, studies of mouse erythrocytes revealed that FP causes hemolysis by inhibit-

ing the ability of biological membranes to maintain cation gradients (10, 11). These studies yielded no evidence of peroxidation of phospholipids in the membranes of erythrocytes lysed by FP (11). In 1982, the toxicity of FP for erythrocyte membranes was confirmed by a group of scientists in Israel (12), but they too provided little information about the molecular basis of the toxicity.

There is now evidence that FP binds to phospholipids (13). This observation is of particular interest because mefloquine binds with high affinity to phospholipids (14). It is reasonable, therefore, to propose that membrane phospholipids are the targets of FP, the chloroquine-FP complex and mefloquine.

APPROACH TO THE PROBLEM

Although this contract had the five technical objectives listed in the abstract, a determination was made early in the course of the work to focus on the biochemical basis of ferriprotoporphyrin IX (FP) toxicity and on FP detoxification. Given the putative central role of FP in the mode of action of chloroquine and related antimalarials, we reasoned that new knowledge in these areas offered the greatest promise of permitting us to accomplish the other four objectives. Experiments therefore were designed to evaluate

1) the interactions of FP, mefloquine, chloroquine and related drugs with biological membranes,

2) the state of FP in malaria pigment, and

3) the possibility that hydrogen peroxide in parasitized erythrocytes would detoxify FP.

REVIEW OF WORK SUPPORTED BY THIS CONTRACT

During the 5 years this contract was active, eight manuscripts were published. Since those papers may be consulted when detailed information about a subject is desired, this review will be an interpretative synthesis of the work.

Because ferriprotoporphyrin IX (FP) may mediate the antimalarial action of chloroquine, we conducted an extensive evaluation of the interactions of FP, chloroquine, mefloquine, and diverse other membrane-active agents on cell membranes, using the erythrocyte as the experimental cell. A description of this work was published in the Journal of Pharmacology and Experimental Therapeutics and is summarized in the following abstract:

> "Because ferriprotoporphyrin IX (ferriheme, FP) is a lytic agent that can be released by degradation or oxidative denaturation of hemoglobin, we measured the hemolytic responses of human erythrocytes to FP alone or to FP in combination with various membrane-active agents. Suspensions of erythrocytes (0.5%) incubated at pH 7.4 and 37° C were hemolyzed by FP alone in concentrations of 10 μM or greater. Preincubation of the erythrocytes with nonhemolytic concentrations of

chloroquine, mefloquine, quinine, calcium, lanthanum or manganese potentiated the hemolytic response to FP. For example, hemolysis in the presence of 5 μM FP was 5%; in the presence of 5 μM FP and 20 μM chloroquine, hemolysis exceeded 80%. For 5 μM FP, maximal potentiation was obtained with 20 μM chloroquine, 200 μM quinine or 1 mM calcium. Paradoxically, with 5 μM FP and a high concentration of chloroquine (1 mM), hemolysis did not exceed the base-line value of 5%. In addition, all of the agents that individually potentiated the hemolytic response to 5 μM FP also inhibited hemolysis when used in combination with 5 μM chloroquine and 5 μM FP. Detailed studies of calcium showed that it inhibited the incease in osmotic fragility, which was induced by FP and chloroquine, without inhibiting the increase in binding of [^{14}C]chloroquine to erythrocytes treated with FP. These results can be rationalized by proposing that the structure of the erythrocyte membrane at the time of exposure to FP and membrane-active agents determines whether the hemolytic response will be potentiated or inhibited."

Additional experiments described in the 1984 Annual Report for this Contract indicate that FP functions as an amphipathic agent and affects membranes in the same way as various detergents. Our working hypothesis is that FP binds to membrane phospholipids, increasing the membranes' fluidity, and eventually causing sufficient pertubation of structure to allow intracellular contents to leak out. It is interesting that antimalarial drugs may potentiate or reduce cell lysis by FP, depending on experimental conditions. However, under most conditions the interaction between FP and antimalarial drugs is much more toxic than the sequestered FP of malaria pigment. Thus, if an interaction prevented FP from being sequestered, it would allow FP to exhibit its intrinsic toxicity. As the foregoing studies were being done, we decided to see whether or not FP is released in erythrocytes that are known to provide hostile environments for malaria parasites, i.e., sickle cells and G6PD-deficient erythrocytes. This work is summarized in the following two abstracts:

"In an effort to elucidate a mechanism of genetic resistance to malaria, we asked whether a toxic form of heme is included in the excess of ferriprotoporphyrin IX (FP) which has been reported to accumulate as hemichromes in sickle cells. When FP is bound to certain erythrocytic elements, such as native hemoglobin, it is inaccessible to bind chloroquine with high affinity and is nontoxic. However, when FP is accessible to bind chloroquine with high affinity, it has been demonstrated to be sufficiently free to have membrane toxicity and, under certain conditions, to lyse Plasmodium falciparum parasites. [^{14}C]-chloroquine was used, therefore, as a reporter molecule to evaluate the quantity, accessibility, and potential toxicity of FP released from hemoglobin. Intact erythrocytes from subjects with sickle cell anemia bound approximately 71 μmoles of chloroquine per kg with an apparent K_d of 10^{-6} M. Erythrocytes from

normal subjects or subjects with sickle trait bound little or no chloroquine with high affinity. Since the oxidant stress introduced by malaria parasites would increase the tendency for denaturation of hemoglobin S with additional release of FP, we suggest that FP toxicity accounts for the death of malaria parasites in sickle cells."

"Hemoglobin in glucose-6-phosphate dehydrogenase (G6PD)-deficient erythrocytes is abnormally vulnerable to oxidative denaturation, which may release ferriheme [FP], a known cytotoxic agent. We found 13.3 nmol of ferriheme in G6PD-deficient erythrocyte membranes (per gram of total erythrocyte hemoglobin) using a spectrophotometric assay, as compared to 9.8 in normal membranes ($P < .05$). After incubation of erythrocytes with 250 µmol/L menadione, an oxidant drug, the values increased by 37.4 nmol in G6PD-deficient membranes and by 26 in normal membranes ($P < .005$), indicating increased hemoglobin denaturation. To verify that hemoglobin ferriheme in G6PD-deficient erythrocytes releases ferriheme in a form available to interact with other ligands, [^{14}-C]-chloroquine binding to intact erythrocytes was measured. With an initial concentration of 5 µmol/L chloroquine in a medium containing no menadione, an excess of 14.8 nmol of chloroquine was bound in G6PD-deficient erythrocytes (per gram of hemoglobin) as compared to normal erythrocytes ($P < .005$). In the presence of 250 µmol/L menadione, chloroquine binding increased by 17.9 nmol in G6PD-deficient and by 7.2 in normal erythrocytes ($P < .005$). These results indicate that ferriheme becomes available to interact with endogenous ligands and, thus, to mediate menadione-induced hemolysis in patients with G6PD deficiency. Furthermore, the increase in ferriheme may mediate the selective toxicity of menadione for Plasmodium falciparum parasites growing in G6PD-deficient erythrocytes. Ferriheme release in response to the intraerythrocytic oxidant stress introduced by malaria parasites also may account for the resistance to malaria afforded by G6PD deficiency."

Next, we focused on the process of FP detoxification. Initially, we searched exhaustively for a heme binding substance that would bind tightly enough to FP to account for its sequestration in malaria pigment. None was found. Then, we decided to evaluate the state of FP in malaria pigment, thinking that this knowledge would allow us to design experiments to elucidate the process of FP sequestration. A description of this work was published in the Journal of Biological Chemistry. The following quotation is a summary of the manuscript.

"To evaluate the state of ferriprotoporphyrin IX (FP) in malaria pigment, mouse erythrocytes infected with Plasmodium berghei NYU-2 parasites were lysed by hypotonic shock, and hemoglobin and other soluble material were removed by extensive washing. The amount of FP recovered in the insoluble pellet was 2.1 µmoles

per ml of packed, infected erythrocytes, of which approximately one percent was attributable to hemoglobin contamination. This crude preparation then was digested with a nonspecific protease from Streptomyces griseus and extracted with chloroform/methanol. The residue of insoluble dark brown material had the spectral and solubility properties characteristic of the FP of malaria pigment, and various different preparations contained from 80 to 99 percent of FP by weight. By elemental analysis, highly purified preparations contained no chlorine and had an oxygen content consistent with one mole of hydroxyl per mole of FP (oxygen content, calculated, 12.6 percent; found 12.5 percent). In comparison to hematin purchased from Sigma Chemical Company, which had a measured oxygen content of 14.7 percent, the low oxygen form of hematin purified from malaria pigment was remarkably less soluble in ethanol, 3 percent sodium bicarbonate, and chloroform."

The ability to sequester FP in an insoluble, nontoxic state is an adaptation that permits malaria parasites to digest hemoglobin without committing suicide. Furthermore, since the process of conversion of FP to an insoluble form of hematin apparently determines the concentration of FP available to bind chloroquine, it may determine whether the parasite is susceptible or resistant to chloroquine. This process therefore requires further study. It will be especially important to determine whether the conversion occurs spontaneously or requires enzymatic intervention.

In addition to sequestration as an insoluble hematin, FP may be detoxified in other ways. A role for hydrogen peroxide as a detoxifying agent is discussed in the manuscript that was published in Life Sciences. Its abstract is given below:

"Two potentially lytic substances, ferriprotoporphyrin IX (FP) and hydrogen peroxide, may coexist and partially detoxify each other in sickle cells and in erythrocytes infected with malaria parasites. Since hydrogen peroxide can decompose FP, its effect on hemolysis induced by FP and by the complex of FP with chloroquine was investigated. Human erythrocytes suspended at a concentration of 0.5% in a 50 μM solution of FP underwent approximately 42% hemolysis during the course of 2 hours. Twenty-five micromolar chloroquine potentiated hemolysis to 99%, and preincubation of 50 μM FP with 25 μM hydrogen peroxide for 5 minutes reduced hemolysis to 4%. Mixing either FP or hydrogen peroxide first with chloroquine abolished the effect of hydrogen peroxide. Detoxification of FP by hydrogen peroxide may be an important protective mechanism in certain hemolytic anemias, and inhibition of detoxification could account for the effectiveness of chloroquine in malaria."

Since hydrogen peroxide exists in parasitized erythrocytes and can decompose FP, we now modify our hypothesis to indicate that malaria parasites can detoxify FP both by sequestering it

and by decomposing it. In the latter case, chloroquine would cause the accumulation of a toxic chloroquine-FP complex by delaying the decomposition of FP by hydrogen peroxide. Finally, we recognize that malaria parasites could limit the accessibility of FP to chloroquine by increasing either sequestration or decomposition. Thus, increased availability of hydrogen peroxide could cause chloroquine resistance in malaria.

CONCLUSIONS

Ferriprotoporphyrin IX (FP) causes cell lysis by binding to membrane phospholipids, increasing membrane fluidity, and eventually causing sufficient disorganization of the membrane that intracellular contents escape. Antimalarial drugs can enhance or reduce the deleterious interaction of FP with cellular membranes, depending on experimental conditions. Under most conditions, the combination of antimalarial drug and FP is more toxic than is the FP sequestered in malaria pigment.

FP is released in sickle cells and G6PD-deficient erythrocytes and probably functions as an endogenous antimalarial agent in these cells.

FP is sequestered in malaria pigment as an insoluble, low-oxygen form of hematin.

FP is detoxified by hydrogen peroxide and the effect of hydrogen peroxide is inhibited by chloroquine.

RECOMMENDATIONS

1. The process of FP sequestration into an insoluble, low-oxygen form of hematin in malaria parasites should be fully characterized.

2. The extent to which hydrogen peroxide detoxifies FP in malaria parasites should be determined.

3. Drugs targeted to the process of FP sequestration and to FP detoxification by hydrogen peroxide should be designed.

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