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PROTECTIVE MECHANISMS AGAINST MALARIA, ASSOCIATED WITH GLUCOSE 6-PHOSPHATE DEHYDROGENASE (6GPD) DEFICIENCY

Annual scientific Report submitted by J. Golenser, M. Chevion and J.L. Ngu - Principal Investigators, March 1990

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a) Papers suported by the grant

1) Oxidant stress and malaria: host-parasite inter-relationships in normal and abnormal erythrocytes (Seminars in Hematology, 1983).

2) Deleterious synergistic effects of ascorbate and copper on the development of *P. falciparum*, an in vitro study in normal and in 6GPD-deficient erythrocytes (International Parasitology, 1989)

b) Report on the visit and meetings of J. Golenser and M. Chevion with Prof. J.L. Ngu in Yaounde, Cameroon.

INTRODUCTION

Malaria, a disease caused by various species of *Plasmodium*, mostly by *P. falciparum*, is a major health problem in many developing nations. More than 400 million people suffer from the disease resulting in over 1.5 million annual deaths. Currently, the control of malaria has been increasingly hampered by the emergence of parasites resistant to the standard antimalarials. One approach to the prevention and therapy of malaria is to follow natural defense mechanisms. These are probably based on reactive oxygen species (ROS) originating in the parasitized erythrocyte or produced by polymorphonuclear neutrophils (PMNs) and macrophages. In certain geographic regions, natural selection has emphasized this principle; positive selection has led to relatively high frequencies of genetically variant erythrocytes- glucose-6-phosphate dehydrogenase deficiencies (G6PD), heterozygosity for hemoglobin S, thalassemias, hereditary persistence of fetal hemoglobin, and hemoglobin E. All of the above erythrocytes are particularly sensitive to oxidant stress and cannot tolerate parasite-induced oxidation.

Malarial plasmodia constitute a typical example of intracellular parasitism. As with all host cell-parasite interactions, these consist of a two-way relationship - the effects of the parasite on the host cell and the effects of the host cell on the parasite. Alteration in this relationship can affect any stage of the development of the parasite including attachment to the

erythrocyte, penetration (or internalization), intracellular development, and the erythrocyte burst-releasing merozoites ready to invade other red blood cells (RBCs).

The suggestion that some disorders confer resistance against malaria has been based on population studies, clinical investigations, and on laboratory experimental work performed on P. falciparum cultures. The role of ROS has also been investigated in vivo in rodent models.

Oxidation processes throughout the development of malaria parasites either in normal or abnormal erythrocytes is a complex subject, and is further complicated by the species diferences and by different conditions of growth. In a heterozygous host for a particular trait, parasites may face erythrocytes that are supportive as well as others rendered nonsupportive under conditions of oxidant stress. The oxidant stress could be derived from immune reactions, drugs, or food constituents. However, the parasites survive in hostile and changing environments by adapting to varying conditions in the host.

GENETIC ASPECTS

Malaria parasites and their human host may affect genetically each other. Two genetic aspects were investigated: a) antigenic definition of some African isolates of Plasmodium falciparum and b) estimation of the prevalence of G6PD deficiency in Yaounde, Cameroon.

a) SEROTYPING OF AFRICAN ISOLATES OF P. FALCIPARUM USING MONOCLONAL ANTIBODIES

The ever-increasing problem of drug resistance shown by malarial parasites has given the development of new drdugs and a vaccine top priority. Information on antigenic diversity within parasite populations is essential for defining various isolates. A serotyping indirect immunofluorescent test, has been performed, to demonstrate serological diversity amongst isolates of Plasmodium falciparum from different endemic regions. Results of a study involving monoclonal antibodies raised against two isolates from Papua, New Guinea, (Schofield et al., 1982) suggested that parasites from a given geographical region are antigenically similar. In this study we used monoclonal antibodies raised against isolates from Thailand, prepared by Dr. J.S. McBride from the University of Edinburgh. We sereotyped 19 culture-adapted isolates from Mozambique, using a panel of nine monoclonal antibodies.

Each of the african P. falciparum isolates reacted with at least four of the monoclonal antibodies raised against isolates of P. falciparum from Thailand. This lends further support to the view that antigenic determinants are shared by isolates from different parts of the world. The eight P. falciparum monoclonal antibodies revealed considerable diversity within this group of african isolates and seven different patterns of reaction were observed. The positive reaction of all the african isolates with

monoclonal antibody 9.8 was to be expected as this specificity has been detected in over 200 culture-adapted isolates from all over the world.

The anti P. berghei monoclonal antibody, D5, has been successfully used to detect Plasmodium in infected blood using a radioimmunoassay. The ubiquitous response of the african strains to this antibody indicates that it is suitable for general use in the immunodiagnosis of malaria in this region.

The heterogeneous response of sixteen isolates to the monoclonal antibodies may be ascribed to the presence of more than one antigenically distinct clone in each isolate. Similar heterogeneity was observed within this same group of african isolates with respect to in vitro chloroquine sensitivity.

Thirteen of the african isolates characterized in this study reacted to a limited degree with both sets of antibodies in the first test. This suggests that at the time of preparation of antigen slides, these isolates consisted of mixtures of organisms expressing alternative forms of the antigen. By the time schizonts were harvested for the third test, nine of these isolates contained a majority of schizonts which reacted with antibodies 7.3 and 7.6 but completely lacked parasites reacting with antibodies 9.2 and 10.3. It is possible that those clones possessing antigenic markers for antibodies 7.3 and 7.6 were able to withstand the freezing/thawing process and/or the culture conditions while those possessing the alternative form were not. This could hae resulted in the loss of organisms specific for

monoclonal antibodies 9.2 and 10.3 in culture. Four isolates possesssed both sets of antigenic determinants during the entire test period. Only one of these was cryopreserved during the study period.

Only two of the 19 african isolates were found to be non-reactive to monoclonal antibodies 7.3 and 7.6. Furthermore, none of the 13 clonally heterogeneous isolates lost the antigenic markers for these antibodies. This was in contrast to a study of seven cultured isolates from Brazil which revealed that they were all negative for monoclonal antibodies 7.3 and 7.6 and positive for antibodies 9.2 and 10.3. Twelve out of 17 Thai isolates were found to be specific for antibodies 9.2 and 10.3 and one isolate reacted with both sets of antibodies. It is possible that through natural selection, isolates from a given geographical region may tend to develop towards a basic antigenic type. However, these various studies were carried out using isolates which had been maintained _in vitro_ under different conditions, in different laboratories, each of these artificial environments being vastly different from the _in vivo_ situation. The culture conditions in a given laboratory may have resulted in the selection of antigenically similar organisms in the laboratory. Seventy percent of all isolates tested in the present study exhibited an identical reaction pattern at the end of the test period after having been passaged many times in culture and, in some cases, after they had been subjected to at least one cryopreservation and thawing procedure.

Ideally, characterization studies should be carried out on primary isolates and their clones, in order to determine the antigenic constitution of the wild population of P. falciparum. A panel of monoclonal antibodies raised against isolates from various endemic regions of the world might be used to answer the question of regional variation within the worldwide population of P. falciparum.

b) PREVALENCE OF G6PD DEFICIENCY IN YAOUNDE, CAMEROON

1) Screening for G6-P-D deficiency for estimation of its prevalence and for a source of individuals bearing deficient erythrocytes for cultures.
2) Screening for some hemoglobinopathies: Thalassemia, and HbS.
3) Establishing in vitro cultures of P. falciparum.

1) Of 52 samples screened for G6PD activity, 17.3% were G6PD deficient. The Bio-Merieux Enzyline method and kit that was used for this work yielded different levels of G6PD activity for each subject. The values obtained ranged from 0.36 units per gram of hemoglobin (0.36u/g Hb) to 3.59u/g HB, representing 8.6% to 81.59% of the Normal.
2) Eighty five (85) students of the Medical School of the University of Yaounde have been screened for G6PD deficiency. HbS, and 31 of these for Thalassemia. G6PD activity ranged from 0.20-6.65u/g Hb. 16 of the 85 subjects (18.8%) were AS.

Percent HbA_2 (done on chromatography using DE52) ranged from 1.84% to 10.89%. 9 out of 31 had values greater than 4%. This is going to be continued up to a minimum of 150.

3) It has been dificult so far to establish viable continuous cultures of P. falciparum using infected blood obtained locally. This, in some cases may be attributed to antimalarial taken sporadically by subjects in Cameroon or to antibodies or crisis form factors in the serum. To obviate this possibility we are now looking for donors who have not been on any medications and had not been recently infected with malaria.

OXIDANT STRESS AND MALARIA

One approach to the prevention and therapy of malaria is to follow natural guidelines. The major natural defense mechanisms are probably based on reactive oxygen species (ROS) originating in the parasitized erythrocyte or produced by polymorphonuclear neutrophils (MPN) and macrophages. In certain geographic regions, natural selection has emphasized this principle. Positive selection has led to relatively high frequencies of genetically variant erythrocyte glucose-6-phosphate dehydrogenase deficiencies (G6PD), heterozygosity for hemoglobin S, thalassemias, hereditary persistence of fetal hemoglobin and hemoglobin E. All the above erythrocytes are particularly sensitive to oxidant stress and parasite-induced oxidation.

a) THE EFFECT OF FREE RADICALS INDUCED BY PARAQUAT AND COPPER ON THE *IN VITRO* DEVELOPMENT OF *PLASMODIUM FALCIPARUM*

It has been previously shown that in *E. coli* system, paraquat toxicity is enhanced by redox transition metals, iron and copper. In this study we used cultures of *Plasmodium falciparum* as a biological indicator for evaluating the role of the transition metals in paraquat toxicity. It is assumed that in this system, the infected erythrocytes "provide" the iron: during the development of the parasite, as hemoglobin is digested a concurrent release of high levels of iron takes place within the red blood cell. In this study, we have chosen to use adventitious copper as an exogenous metal in order to allow discrimination between the effects of the endogenous and added metals. Additionally, copper *per se*, is known to act as an oxidant agent on red blood cells.

We have found that copper itself is toxic to the parasites. 16uM of copper added to parasitized red cells killed most of the parasites. 2uM copper inhibited the incorporation of hypoxanthine (Hx) by the parasites by approximately 17%. Treatment with 2uM copper in an oxygen depleted atmosphere caused only 2-5% inhibition. Etkin and Eaton had showed that parasitized erythrocytes release H_2O_2. In the presence of copper the Fenton reaction can take place and free radicals can be generated, and may affect the non-infected cells in the culture. This conclusion

is supported by the fact that pretreatment of normal RBC before infection with even a higher copper concentration, i.e. with 3uM did not inhibit neither invasion nor the intracellular growth of the parasites at all. In contrast, this concentration caused 46% inhibition in pretreated G6PD(-) cells. These findings are consistent with those of Calabrese who showed a markedly increased sensitivity in G6PD(-) cells which was expressed by the accumulation of copper-induced methemoglobin and decreased levels of acetylcholinesterase activity, as compared to normal red blood cells.

Inhibition of Hx uptake following treatment of parasitized erythrocytes with paraquat was dose and time dependent. The combination of copper and paraquat yielded an additive effect, whereas it was synergistic in a bacterial system. The effects of paraquat and copper in a mixture of non-infected and infected cells may be directed against different cellular targets and therefore would be only additive. When we eliminated one of the targets by either treating parasitized red blood cells that were only later diluted with untreated NRBC or pretreating uninfected red blood cells before the addition of parasites, the effect of copper and paraquat was synergistic.

The role of transition metals in the enhancement of oxidant stress was further examined by the addition of the chelators diethylenetriamine pentaacetic acid (DTPA) and desferrioxamine (DFO) to the experimental system. These chelators reduced the inhibition of plasmodial growth that had been imposed by

paraquat, copper or their combination. The fact that 10uM DTPA and 33uM DFO reduced PQ toxicity almost completely indicate that its activity is dependent on endogenous transition metals. The inhibitory effect of paraquat probably is mediated by the newly released yet undefined form of intracellular iron.

The effect of DTPA and DFO is in accord with the protection provided by DFO in alloxan-induced damage to plasmodia _in vivo_, in mice (Clark and Hunt, 1983). It could also be reconciled with long term exposure to DFO (Fritch and Jung, 1986). There, the chelator deprives the parasite from utilizing iron, which is essential for the parasite growth, and by this, enhances the damage rather than provides the expected protection.

Parasitized G6PD(-) erythrocytes showed only slightly higher sensitivity toward a challenge of paraquat, than the parasitized normal erythrocytes. Since the production of the free radicals induced by paraquat is NADPH dependent, and since G6PD(-) parasitized cells have much less NADPH under oxidant stress, this result is not surprising, despite the general higher sensitivity of G6PD(-) erythrocytes to oxidant stress. However, the increased sensitivity of G6PD(-) erythrocytes was expressed, as was mentioned above, in the other experiments where non-infected erythrocytes were exposed to the challenge and only subsequently were infected with _P. falciparum_. In these experiments G6PD(-) erythrocytes were markedly more sensitive toward the challengers, paraquat and in particular copper and their combination.

The results in this study suggest that the deleterious effects of paraquat are metal-dependent and occur via a free radical pathway. The synergism between paraquat and copper substantiates this suggestion. Our understanding of the role of transition metals, reducing agents and free radicals in parasite development and injury provides novel strategies in the fight against malaria.

b) MECHANISMS RESPONSIBLE FOR THE DESTRUCTION OF P. FALCIPARUM BY ASCORBIC ACID AND COPPER

Ascorbate can act both as an antioxidant and as a prooxidant agent. Its antioxidative effect was noticed either after pretreatment of uninfected cells or following treatment of red blood cells infected with young ring forms. On the second day of the cycle, when the parasites were developing from ring to mature form in the erythrocyte and most of the hemoglobin had already been digested, the effect of ascorbate could be attributed to its increased iron-mediated prooxidant potency. The effect of ascorbate as a proxidant was very much enhanced by copper and indicated the involvement of the hydroxyl radical in this process. Addition of DTPA decreased the effect of the copper and of the endogenous transition metals.

The deleterious synergistic effects of ascorbate and copper suggest that the effects are transition metal dependent, and that they occur via a free radical pathway process. We tried to

evaluate the mechanism by which the parasite is affected. Ascorbate exerted an oxidative stress which resulted in the release of oxygen reactive species, detected by HPLC (high performance liquid anion exchange chromatography). We used salicylic acid as a scavenger of hydroxyl radicals. Salicylic acid, which is hydroxybenzoic acid, is converted by hydroxyl radicals to dehydroxybenzoic acid which can be detected by the HPLC. Uninfected NRBC and parasitized RBC containing mature parasites grown in erythrocytes from the same donor, were studied. These cells were incubated for 1 hour at 37°C with or without ascorbate, in the presence of salicylic acid. The supernatant and pellet were frozen, thawed, and then examined in the HPLC. More hydroxyl radical was produced by the parasitized cells than by NRBC. The parasitized cells treated with ascorbate produced more hydroxyl radicals than NRBC treated in the same manner.

Ascorbate and copper caused the oxidation of hemoglobin to methemoglobin. This oxidation was more pronounced in parasitized than in non-parasitized cells, even without oxidants.

The treatment with copper and ascorbate induced peroxidation of membrane lipids measured by the production of malondialdehyde (MDA). Ascorbate alone led to the production of small amounts of MDA, but the combination of ascorbate and copper in parasitized G6PD deficient cells led to synergistic production of MDA.

The role of erythrocyte enzymes in protecting the parasites against oxidative damage is demonstrated in experiments using

free plasmodia (released by saponin treatment from the host cell). Free plasmodia were more vulnerable to the oxidative challenge than parasites within erythrocytes (viability was measured by Hx incorporation). This is in accord with the assumption that erythrocytic enzymes such as catalase, peroxidase and SOD provide the intracellular parasite with some degree of protection from external oxidation. The assumption that the damage was inflicted on the intraerythrocytic parasite by oxidative stress was further supported by the fact that the addition of extracellular catalase interfered with the effects of ascorbate.

We may conclude that the destruction of the parasites is transition metal dependent via a free radical pathway. The damage to the parasite is caused by several modes of interaction at different sites of contact between the parasite and the host cell.

c) INDUCTION OF OXIDANT STESSS BY AVAILABLE IRON AND REDOX ACTIVE FORMS IN ADVANCED STAGES OF PLASMODIUM FALCIPARUM

Oxidative stress has been incriminated as a deleterious factor in the development of malaria parasites. Various chemical reductones which can undergo cyclic oxidation and reduction, such as ascorbate have been shown to cause oxidative stress to red blood cells. Ascorbate, the naturally-occurring and redox-active compound, can induce the formation of active oxygen derived

species, such as superoxide radicals ($\cdot O_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH\cdot$). The formation of the hydroxyl radical, the ultimate deleterious species, is mediated by the redox-active and available transition metals iron and copper in the Haber-Weiss reaction.

During the development of the parasite, hemoglobin is progressively digested and a concurrent release of high levels of iron-containing breakdown products takes place within the red blood cell. Indications for the progressive increase in redox-active iron during the growth of P. falciparum have been recently found in our laboratory: a) adventitious ascorbate proved highly detrimental to the parasite when added to the mature forms. In contrast, if the parasitized erythrocytes were in the early phase following invasion, and only low levels of iron-containing structures had been liberated, then the observed effect was a small enhancement of parasite development. b) erythrocytes containing advanced stages of the parasite were much more active than erythrocyters containing young ring forms as a source for redox-active iron in the ascorbate-driven metal-mediated degradation of DNA. The addition of extracts from parasitized erythrocytes and ascorbate to DNA caused a dose and time dependent DNA degradation. Non-infected erythrocytes had no effect. These findings could also propose that the parasite-dependent accumulation of redox-active forms of iron within the erythrocytes serve as a biological clock triggering the rupture

of the red blood cell membrane at the right moment, when the parasite reaches its maturity.

d) SHOULD VITAMIN C BE USED FOR CONTROL OF MALARIA?

Vitamin C can act both as an anti-oxidant and as a pro-oxidant. The pro-oxidant effect of ascorbate is clearly seen in the presence of redox-active iron or copper.

During its development, the malaria parasite, *Plasmodium falciparum*, digests erythrocyte proteins - mostly hemoglobin. In spite of the special care that the parasite practice in handling heme and other iron-containing structures that are released from the degraded hemoglobin, there is leakage of iron-containing structures within the red blood cell, rendering the parasite highly susceptible to reducing agents. These ideas were examined, *in vitro*, in human blood, when *P. falciparum*-infected cells were exposed to ascorbate. The mature forms of the parasite (when 50%, or more, of the hemoglobin had already been degraded) proved very sensitive to ascorbate, in a dose-dependent mode. It is assumed that when a population of erythrocytes is exposed to vitamin C, an intensive pro-oxidant reaction will occur only within the infected cells. At the same time non-infected cells. will enjoy the anti-oxidant action of ascorbate. This will induce selective damage within infected cells, and will cause specific inactivation of the parasites.

Further substantiation of this idea was obtained when P. berghei-infected Balb/c mice were injected with vitamin C. During treatment with ascorbate the level of parasitemia remained at 50% (or less) of non-treated mice. Thus, vitamin C alone, or in combination with conventional anti-malarial drugs, should be further examined for its possible use in malaria control.

e) ADAPTATION OF PLASMODIA TO OXIDANT STRESS

In the case of thalassemic or G6PD-deficient erythrocytes, which are already susceptible to ROS, the invasion of such a cell by a parasite producing more oxygen radicals could prove fatal for it. The parasite is not indifferent to the oxidant damage threatening its survival and tries to adapt itself.

We found a reduction in parasitemias of P. falciparum in G6PD-deficient cells originating from both decreased invasion and retardation of intracellular development, in comparison with normal erythrocytes. While it has been generaly accepted that the parasite does not synthesize its own G6PD and utilizes the host protein, we found an electrophoretically slow moving plasmodial protein with G6PD activity. Usanga and Luzzato suggested that when the parasites develop in G6PD-deficient erythrocytes, they undergo adaptive changes to activate their G6PD gene and to produce their own enzyme. This enables them to circumvent the unfavorable conditions presented by the abnormal host cell, and to develop in the deficient cell as in the normal one.

We cultured P. falciparum for 50 cycles in G6PD-deficient erythrocytes and challenged the parasitized erythrocytes with ascorbate and copper, which exerted oxidative stgress. The results suggest that our variant has adapted to the deficient RBC but demonstrates only moderate enhancement of resistance to oxidative stress in G6PD deficient cells. The parasite adaptation may, therefore, be sufficient to compensate for only minor changes of oxidative stress. There are other variations in relation to the theory of adaptation: we have found at least one variant of P. falciparum (VNS) that cannot adapt itself to developing within G6PD-deficient erythrocytes. It is important, therefore, to examine different variants of P. falciparum in an area where malaria is endemic and where G6PD deficiency is ubiquitous, in order to evaluate further the hypothesis of Usanga and Luzzato.

f. THE ROLE OF PMNs IN THE DESTRUCTION OF MALARIAL PARASITES

ROS generated by macrophages and PMNs play an important role in the host defence against malaria.

Non-activated human PMNs were added to cultures of P. falciparum in microtitre cells. Radioactive hypoxanthine was added for the estimation of the viability of the parasites. This nucleotide is incorporated only by the plasmodia, and not by the leucocytes. At a PMN/RBC ratio of 1/150 (starting parasitemia was 3%) parasite incorporation was 65% of the control cultures. G6PD

deficient parasitized erythrocytes were more sensitive to the PMNs than normal parasitized erythrocytes, by about 25%. This small (but significant) difference could be attributed to the production of reactive oxygen intermediates in the experimental system: PMNs exerted oxidative stress which resulted in the release of oxygen-reactive species detected by HPLC. We used salicylic acid as a scavanger of the hydroxyl radical (the most damaging radical). Salicylic acid, which is hydroxybenzoic acid, is converted by hydroxyl radicals to dihydroxybenzoic acid, which can be detected by the HPLC. Uninfected NRBC or parasitized erythrocytes containing young ring forms could not trigger the PMNs to produce ROS, and only the mature forms of the parasites were active. The more pronounced effect on the parasitized G6PD deficient erythrocytes correlates with the generally increased sensitivity of G6PD-deficient erythrocytes to oxidant stress. However other factors associated with PMNs may inactivate the parasites, such as phagocytosis, lysosomal enzymes or degradation toxic products of the PMNs.

Experiments with chemiluminescence as a method of estimating PMNs stimulation show that the active stages of the parasite are the mature schizonts and the merozoites released after segmentation.

Oxidant Stress and Malaria: Host-Parasite Interrelationships in Normal and Abnormal Erythrocytes

Jacob Golenser and Mordechai Chevion

MALARIA, a disease caused by various species of *Plasmodium*, mostly by *P falciparum*, is a major health problem in many developing nations. More than 400 million people suffer from the disease resulting in over 1.5 million annual deaths.[90,104] Currently, the control of malaria has been increasingly hampered by the emergence of parasites resistant to the standard antimalarials.[112] One approach to the prevention and therapy of malaria is to follow natural defense mechanisms. These are probably based on reactive oxygen species (ROS) originating in the parasitized erythrocyte or produced by polymorphonuclear neutrophils (PMNs) and macrophages. In certain geographic regions, natural selection has emphasized this principle; positive selection has led to relatively high frequencies of genetically variant erythrocytes—glucose-6-phosphate dehydrogenase deficiencies (G6PD), heterozygosity for hemoglobin S, thalassemias, hereditary persistence of fetal hemoglobin, and hemoglobin E. All of the above erythrocytes are particularly sensitive to oxidant stress and cannot tolerate parasite-induced oxidation.[111]

From a biological point of view, malarial plasmodia constitute a typical example of intracellular parasitism. As with all host cell–parasite interactions, these consist of a two-way relationship—the effects of the parasite on the host cell and the effects of the host cell on the parasite.[65] Alteration in this relationship can affect any stage of the development of the parasite including attachment to the erythrocyte, penetration (or internalization), intracellular development, and the erythrocyte burst–releasing merozoites ready to invade other red blood cells (RBCs).[27,33]

The suggestion that some disorders confer resistance against malaria has been based on population studies, clinical investigations, and on laboratory experimental work performed on *P falciparum* cultures.[108] The role of ROS has also been investigated in vivo in rodent models.[11,17,18,73,102,103]

Oxidation processes throughout the development of malaria parasites either in normal or abnormal erythrocytes is a complex subject, and is further complicated by the species differences and by different conditions of growth. In a heterozygous host for a particular trait, parasites may face erythrocytes that are supportive as well as others rendered nonsupportive under conditions of oxidant stress. The oxidant stress could be derived from immune reactions, drugs, or food constituents. However, the parasites survive in hostile and changing environments by adapting to varying conditions in the host.

EPIDEMIOLOGICAL AND CLINICAL STUDIES

Epidemiological studies have indicated a geographical correlation between the regions of high incidence of malaria and those where certain inherited disorders of the RBCs are prevalent.[1,2,35,40,63,65,72] In the case of the sickle cell trait, some clinical investigations revealed that persons heterozygous for hemoglobin S (HbS) are more resistant to malaria.[63] Analogous results were reported in females heterozygous for G6PD deficiency.[8]

These findings are in line with the general biological theory that genetic disorders of RBCs are expressed as balanced polymorphisms. Such a balanced polymorphism would need to stem from a selective force in evolution, which could provide an advantage to the heterozygote phenotype. However, various population studies[7a,24a,32,56,59,63] indicate that this correlation is only partial and that in certain areas it is completely absent. Huheey and Martin[45] suggest that G6PD deficiency confers protection against malaria only in those areas where favism is ubiquitous (favism is the occurrence of an acute hemolytic crisis following the ingestion of broad beans in G6PD-deficient individuals).

Contradictory results were reported for HbE,

From the Departments of Parasitology and Cellular Biochemistry, the Hebrew University-Hadassah Medical School, Jerusalem, Israel.

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Address reprint requests to Jacob Golenser, PhD, Department of Parasitology, the Hebrew University-Hadassah Medical School, PO Box 1172, Jerusalem, Israel, 91010.

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and alpha and beta thalassemias.[107] In addition, clinical studies have proven ambiguous because the overall infectious process in endemic areas is masked by physiological, nutritional, and immunological factors, and also by the uncontrolled use of antimalarial drugs. Likewise, Luzzato[65] and Teo and Wong[107] have found the epidemiological data and the clinical studies inconclusive. However, these investigators note that many mutations at various loci of different chromosomes have yielded large numbers of heterozygote variants of a relatively homogeneous phenotype in malarious areas that are geographically distant. Theoretically, they suggest that the genetic traits persist because they provide protection against malaria.

THE MECHANISM OF INTERFERENCE WITH PARASITE DEVELOPMENT

During the course of its development the parasite profoundly alters the structure and function of its host erythrocyte. Most notable are the changes in the membrane, including a marked increase in permeability,[30,61] increased glycolipid fluidity,[43,99] and reduced deformability.[20] Fine structural changes were observed in the cytoskeleton of the infected erythrocytes.[106] These membrane modulations are accompanied by antigenic alterations.[41,46,115]

It can be assumed that these alterations are inflicted on the erythrocyte to support the development and survival of the parasite by allowing the influx of vital nutrients and the secretion of toxic catabolites.[31] An additional effect of the parasite on its host cell is the production of special "knobs" on the membrane of erythrocytes infected with *P falciparum* (Fig 1A). These knobs are responsible for cytoadherence of the infected erythrocytes to endothelial cells, enabling such parasitized cells to avoid removal by the spleen.[41]

With the development of the parasite, the alterations in the cell host are more significant (Fig 2) and oxidant stress induced by the parasite becomes more pronounced. Survival of both parasite and erythrocyte become matters of a delicate balance. Modulations in this state such as a host cell which is more sensitive to oxidant stress, or additional stress originating from external sources (food constituents or drugs) or induced by the immune system may interfere with the successful development of the parasite.

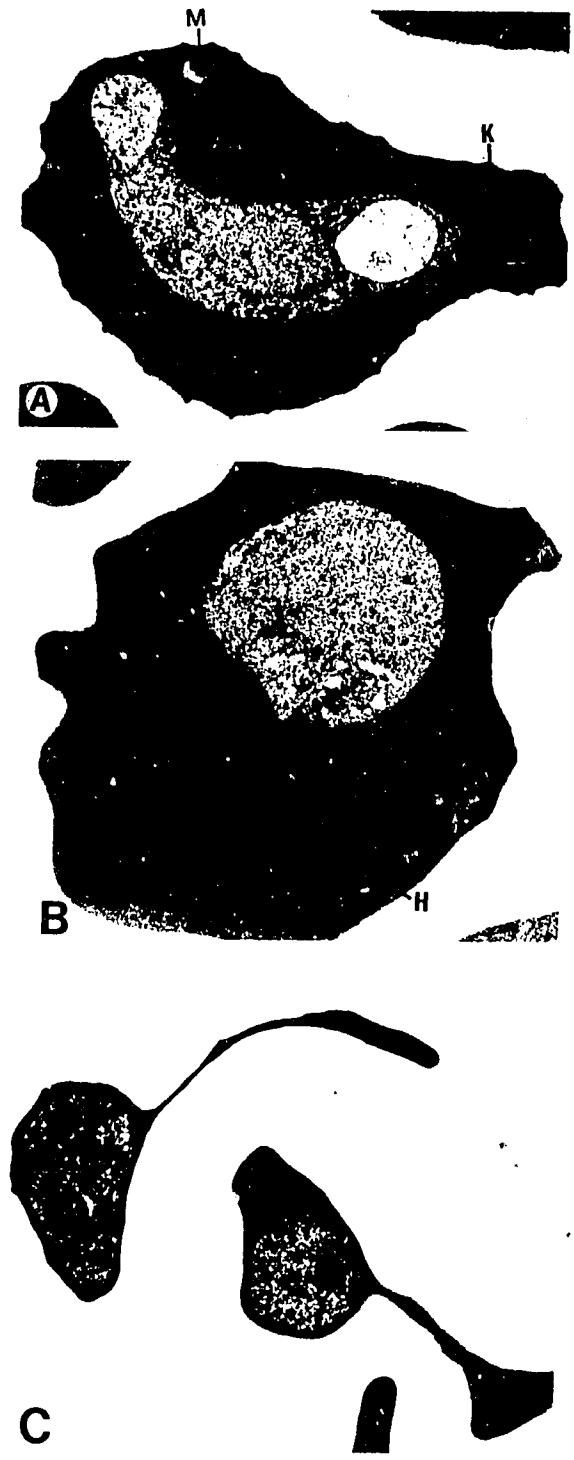

Fig 1. Transmission electron micrographs of *P falciparum* in β-thalassemic erythrocytes. (A) *P falciparum* in normal erythrocytes (original magnification × 14,800). (B) Degenerating parasite in a thalassemic erythrocyte (original magnification × 18,800). (C) Thalassemic erythrocytes infected by *P falciparum* (original magnification × 9,200). The parasites are not developed and there are only very few knobs on the surface of the cells. M, Maurer's clefts; K, knobs; H, Heinz bodies. (Reproduced with permission.[99])

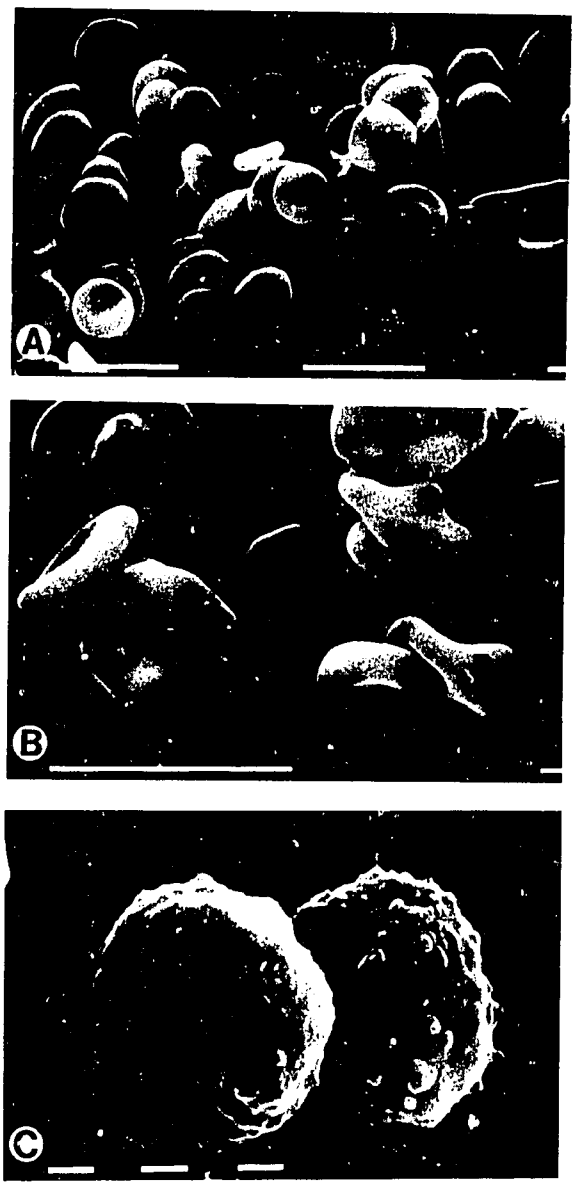

Fig 2. Scanning electron micrographs of erythrocytes infected in vitro with *P falciparum*. (A) Normal erythrocytes (original magnification × 2,100). (B) Erythrocytes containing ring forms (original magnification × 4,200). (C) Erythrocytes containing trophozoites (original magnification × 9,000). (Reproduced with permission.[69])

Structural Factors

It is generally agreed that RBC disorders that are common in malaria-endemic areas confer partial protection against malaria by their abnormally high sensitivity to oxidant stress. However, some other factors affecting the fate of the parasites in such cells should be considered.

RBCs from individuals suffering from inborn errors such as α-thalassemia, β-thalassemia, hemoglobin C (HbC), and HbE in homozygous conditions, show a low mean corpuscular hemoglobin (or mean corpuscular hemoglobin concentration). Luzzato[65] proposed that the smaller erythrocytes, having an excess of membrane with respect to cytoplasmic volume, may physically affect the malaria parasite either by interfering with its development or by facilitating its removal. The retarded developments[49a] can be seen in electron micrographs (Fig 1) of *P falciparum* in such β-thalassemic erythrocytes.[69] It has been suggested that under low oxygen tension the parasites are adversely affected within sickling parasitized cells that are either homozygous or heterozygous for HbS (SS or SA cells), in vivo[65] or in vitro.[25]

Hereditary elliptocytosis (HE) is a heterogeneous group of disorders in terms of inheritance, red cell morphology, severity of hemolysis, and underlying molecular defect. The variants include asymptomatic or symptomatic HE, hereditary pyropoikilocytosis (HPP), spherocytic HE, and the stomatocytic elliptocytosis or Melanesian ovalocytosis. The clinical heterogeneity of the HE/HPP syndrome is paralleled by a difference in the molecular defect within the membrane of these cells. The most common defect, found in ~30% of HE patients and all HPP patients, is an abnormal spectrin dimer (SpD) self-association in which the abnormality is localized on the α_1 domain of spectrin representing the SpD-SpD contact site. Several spectrin variants have been described that result in defective SpD-SpD association into tetramers, producing an unstable and less deformable cell[(23a)]. Erythrocytes from nine donors with HPP were tested for susceptibility to invasion by *P falciparum* merozoites in vitro. The invasion was, in all but one sample, significantly reduced, varying from 38% to 71% of the invasion observed in normal cells. One explanation for the reduced invasion seen in HPP cells relates to the increased SpD within the membrane of these cells (rather than the 30% decrease in spectrin content, also a feature of HPP) and the resulting decreased deformability of the cells.[23a,57] Ovalocytic erythrocytes from Melanesians in Papua, New Guinea, have been demonstrated to be resistant to infection by *P falciparum* in vitro. The epidemiological data in this area also demonstrate enhanced resistance against *Plasmodium vivax* and *Plasmodium malariae*. The ovalocytes

were found to be more thermostable than normocytes, suggesting that there is a major difference in cytoskeletal structure that could affect invasion by all plasmodia.[57]

In homozygous hemoglobin CC (HbCC) cells a membrane alteration with reduced deformability is found. The invasion and development of the parasites through schizogony is identical to that in hemoglobin AA (HbAA) cells under various oxygen tensions. However, there is an interference of the host-cell rupture that prevents release of merozoites and, consequently, the continuity of parasite cell cycle. The interference with parasite-induced RBC lysis is probably caused by the high osmotic resistance of the parasitized CC cells.[76] Olson and Nagel[76] showed that the osmotic resistance does not exist in heterozygous AC cells that maintain normal growth of *P falciparum*. They assert that other mechanisms, such as oxidant stress, may be active in such a state. This combination of structural factors and excessive susceptibility to oxidant stress may be valid for the AS cells or the ovalocytes that have been previously mentioned.

Mortality from malaria is highest during early childhood when acquired immunity has not yet fully developed. At this stage heterozygotes for β-thalassemia have elevated hemoglobin F (HbF).[6] The intracellular development of *P falciparum* in such cells is retarded.[80,82] It is tempting to suggest that the presence of HbF itself may compensate for the lack of immunity in infancy.[107] However, in genetic traits that are not accompanied by increased levels of HbF such as in hemoglobin H (HbH) erythrocytes, there is also impairment of parasite growth.[48] The present study found that erythrocytes from cord blood containing high concentrations of HbF were good hosts for the parasite. It seems, therefore, that the persistence of HbF in certain thalassemic erythrocytes is not directly responsible for the decreased parasitemias, and that oxidant damage mediates the resistance.[69]

Enhanced Erythrocyte Oxidative Modification by Malaria Parasites

The results relating to HbF demonstrate the difficulty of discriminating among the various factors affecting the coexistence of the host cell-parasite relationship. Nonetheless, some effects of the parasite can be resolved and analyzed separately.

Elevated levels of methemoglobin were found in mice erythrocytes infected with *Plasmodium berghei*[23] and in human erythrocytes parasitized with *P falciparum*.[98] On the other hand, Hempelmann et al[38] could not find evidence for increased oxidative modification of hemoglobin in *P falciparum* parasitized cells. As will be discussed later, oxidation may be enhanced in the hemoglobin of genetic variant erythrocytes, especially under additional external pressure.

Lipid peroxidation was reported in membranes of erythrocytes parasitized with *Flasmodium chabaudi*,[73] *Plasmodium vinckei*,[11] and *P falciparum*.[113] An increase in the concentration of malonyldialdehyde, a lipid peroxidation product, was found in the blood of mice infected with *P berghei*.[83] Lipid peroxidation may be involved in the parasite-induced alterations in membrane functions that are crucial for parasite development (see the section on the mechanism of interference with parasite development).

ROS are generated in mice infected with *P berghei*[23] and in erythrocytes infected in vitro with *P falciparum*.[26] The production of increased amounts of ROS by the parasite depletes the erythrocyte of its defense mechanisms—superoxide dismutase (SOD), catalase, glutathione peroxidase, reduced nicotinamide-adenine dinucleotide phospate (NADPH), reduced nicotinamide-adenine dinucleotide (NADH), reduced glutathione (GSH), and glutathione reductase (GR).[111] As oxidative stress is exerted by the growing parasite in cells that are highly sensitive to such a challenge, the enhanced alterations could result in retarded development of the parasite (Fig 3).

Specific Interferences by Exogenous Agents

Genetic traits or fully expressed disorders of the RBC comprise a prerequisite for partial protection of the host against malaria. Hemoglobinopathies or G6PD deficiency alone may not be sufficient to explain the selective pressure exerted on the population that preserves these otherwise often deleterious genotypes. Additional external stress of environmental origin may also affect the persistence of the various polymorphisms.

Interference with parasite development originating from external oxidants is associated with different sites of interaction between the parasite and its host cell; oxidant damage may be expressed either in the host membrane or cytosol,

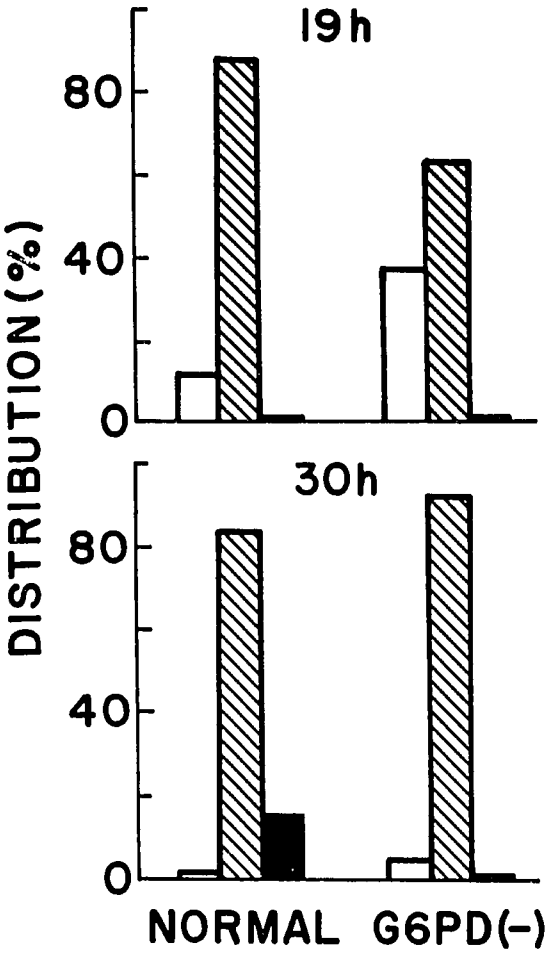

Fig 3. Distribution of developmental stages of parasites growing in normal or G6PD-deficient erythrocytes. □ Ring forms. ▨ Trophozoites. ■ Schizonts. (Reproduced with permission.[34])

or in various organelles of the parasite itself. Pretreatment of G6PD-deficient erythrocytes with compounds inducing oxidant stress[33,70] rendered them unsuitable for merozoite invasion; identical pretreatment of normal cells did not affect parasite growth (Fig 4). Parasite development was affected under conditions in which intracellular GSH was oxidized to glutathione disulfide (GSSG) and membrane intrachain and interchain disulfides were produced.[70] Similar protein profiles were analyzed in membranes of G6PD-deficient cells and in β-thalassemic cells treated with oxidants (E. Hempelmann, personal communication, 1988). Likewise, external inducers of ROS have been shown to cause membrane lipid peroxidation, as measured by the production of malondialdehyde (J. Golenser, E. Marva, M. Chevion, unpublished results). Tumor necrosis serum produced following in vivo nonspecific stimulation with bacillus Calmette-Guéirin (BCG) and lipopolysaccharides (LPS) is parasiticidal to *P falciparum* because of its lipid peroxides.[89] In the process of membrane destruction leading to peroxidation of phospholipids, both iron ions[60] and energy charge play an important role. Adenosine triphosphate (ATP) depletion may induce changes in phospholipid susceptibility toward phospholipases.[29] It is of interest to note that observations of altered phospholipid asymmetry in malaria-infected RBCs are similar to the findings for deoxygenated and completely sickled erythrocytes, older human RBCs cells, and erythrocytes following membrane lipid peroxidations (I.W. Sherman, personal communication, 1988). The changes in the membrane may not only affect the parasite directly at the point of parasite entry, but also may affect the preceding phase of intracellular development by altering the transport of various necessary nutrients.[30,31]

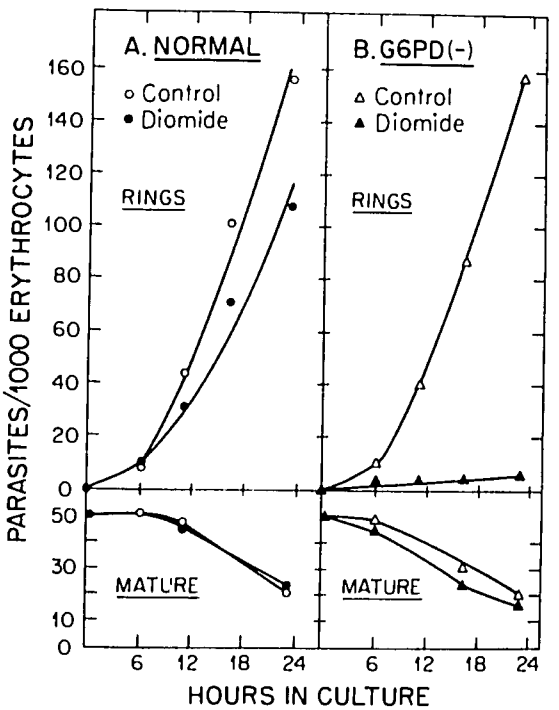

Fig 4. The development of ring forms of *P falciparum* in normal and in G6PD-deficient human erythrocytes pretreated with diamide. Erythrocytes (10% suspensions) were incubated for 30 minutes with or without 1.0 mmol/L diamide, then washed and resuspended in growth medium. Cell suspensions were then inoculated with parasitized erythrocytes (mature forms) to an initial parasitemia of 5% and cultured. Samples were examined every 5 to 6 hours during the first 24 hours of culture. (A) Normal cells. ○, control; ●, diamide treated. (B) G6PD-deficient cells. △, control; ▲, diamide treated. (Reproduced with permission.[70])

The energy status of the cell is also crucial for the parasite because depletion of ATP impairs parasitic development.[10,92]

The parasite itself does not alter the ratio between the concentration of hemoglobin and methemoglobin.[38] However, this ratio is changed when parasitized erythrocytes are treated with oxidants, especially when the host cell is G6PD-deficient (E. Marva, J. Golenser, and M. Chevion, unpublished results[68a]). Abnormal hemoglobins such as hemoglobin S and F appear to autoxidize more readily and to be more sensitive to oxidants than normal adult hemoglobin (HbA).[37,111] Rice-Evans and Baysal[87] suggest that a balance exists between the oxidation state of the hemoglobin and the oxidative deterioration of the membrane lipids, which is dependent on the metabolic state of the erythrocytes.

"Direct hit" of the parasite by oxidant stress has never been shown. However, by analogy to the DNA damage in *Escherichia coli* that is mediated by the Fenton chemistry, DNA damage that is induced by free radicals is possible in this case as well.[47] Bitonti et al[9] found that bis (benzyl) polyamine analogs inhibit the growth of *P falciparum*, possibly by blocking the intracellular function of the natural polyamines in binding sites on DNA. The natural polyamines and their analogs are oxidized by amine oxidase to produce cytotoxic products, including hydrogen peroxide and eventually other ROS. These products can kill intraerythrocytic parasites without lysing the host cell, probably by interference with DNA replication.[71] In spite of the circumstantial evidence for a site-specific damage, its characterization and interpretation are complicated by the variety of direct and indirect effects and by the diversity of the employed oxidative stresses.[87]

The Role of Transition Metals

Oxidative stress has been incriminated as a deleterious factor in the development of malaria parasites. However, the mechanism by which the parasite is affected by such a challenge needs further clarification. Various chemical reductones,[13] which can undergo cyclic oxidation and reduction, such as dialuric acid/alloxan,[15] isouramil,[33] divicine,[18] or butyl hydroperoxide,[17] have been shown to cause oxidative stress to RBCs. Likewise ascorbate, a naturally occurring and redox-active compound, can induce the formation of active oxygen-derived species such as superoxide radicals ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH\cdot$). It is widely accepted that the hydroxyl radical may be the ultimate deleterious species in a variety of biological systems including this one.[12,36] Also it is generally assumed that the formation of $OH\cdot$ is mediated by the redox-active and available transition metals iron and copper.[4,12]

It has been suggested that traces of redox-active and available iron or copper can catalyze the transformation of a superoxide radical anion ($\cdot O_2^-$) to the highly reactive hydroxyl radical ($OH\cdot$), via the metal catalyzed Haber-Weiss reaction.[12,36] During the development of the parasite, hemoglobin is progressively digested and a concurrent release of high levels of iron-containing breakdown products takes place in the RBC. This release is more pronounced in the G6PD-deficient cells,[49] which is in accord with the fact that such cells are more susceptible to oxidative challenge.

Addition of a specific chelator for iron and/or copper, such as desferrioxamine and diethylenetriaminepentaacetic acid (DETAPAC), can remove the metal from its biological binding site and alter its redox potential. However, both of these chelators only sparingly infiltrate into cells.[62] Upon binding, the metal becomes soluble and, if it undergoes redox in its chelated form, it does so away from the essential site. Alternatively, the metal may not be accessible to the reducing agent or hydrogen peroxide.[12] In either case this could prove beneficial to the parasite. In vivo studies using the mouse model have indicated that the level of parasitemia is depressed by the redox-active xenobiotic alloxan.[15] The effect of alloxan could be prevented by prior administration of desferrioxamine. In vitro, long-term exposure to high concentrations of the chelator prevented the parasite from using the iron that is essential to its growth,[77,84] and thus enhanced the damage to the parasite[28] rather than providing the plasmodia with the expected protection.

Indirect indications for the progressive increase in redox-active iron during the growth of *P falciparum* have been recently found. In one series of experiments[68] adventitious ascorbate was shown to promote plasmodial development when given in the early phase following invasion, whereas ascorbate proved highly detrimental to

the parasite when added to the mature forms. In another set of experiments, when infected erythrocytes served as a source for redox-active iron for the ascorbate-driven metal-mediated degradation of DNA, only erythrocytes containing mature parasites were active and caused a concentration- and time-dependent DNA degradation, whereas saponin-purified parasites or noninfected erythrocytes did not. The effect on DNA increased in parallel with the intracellular development of the parasites (E. Marva, J. Golenser, R. Har-El, and M. Chevion, unpublished results[68b]). These findings suggest that the parasite-dependent accumulation of redox-active forms of iron within the erythrocyte could serve as a biological clock triggering the rupture of the RBC membrane at the right moment when the parasite reaches its maturity.

THE ROLE OF THE IMMUNE SYSTEM

Reactive oxygen intermediates, including hydrogen peroxide, generated by macrophages and PMNs, play an important role in the host defense against malaria.[3,53-55,75,105] Vaccination can increase protection by focusing the cytotoxic attack in the spleen and the liver where macrophages are abundant.[22,94,105] Cell-mediated immunity (CMI) to malaria involves ROS release from cells stimulated by γ-interferon and tumor necrosis factor (TNF). These cytokines are produced by T lymphocytes from malaria patients in response to *P falciparum* antigens,[88,109] and can inhibit malarial development in vivo.[16,67] Clark et al[16] have also found enhanced parasitemias in mice that were fed butylated hydroxanisole, a lipid-soluble free radical scavenger.

Extensive discussion of various aspects relating to the relationship between CMI and oxidant damage is beyond the scope of this review. However, it is important to mention the relationship between CMI, oxidant damage, and the pathology of malaria. The same mechanisms that lead to destruction of the parasite or the parasitized cell may prove disadvantageous to the host. For example, TNF, which is secreted during malaria infection, may protect against the parasites but is, on the other hand, a key mediator of endotoxicity and inflammation. Thus, there are many pathological phenomena that are attributable to malaria and the accompanying TNF toxicity[14,14a] (Table 1[114]).

Table 1. Changes Common to TNF Toxicity and Human Malaria

Headaches	Dyserythropoiesis
Fever, chills, and rigors	Hypertriglyceridemia
Myalgias	Elevated blood lactate
Nausea	Renal tubule necrosis
Thrombocytopenia	Pulmonary neutrophil margination
Hypotension	Pulmonary edema
Hyperinsulinemia	Adrenal necrosis
Hyperglycemia, then hypoglycemia	Gut congestion and diarrhea
Erythrophagocytosis*	

Abbreviation: TNF, tumor necrosis factor.

*Common to human malaria and endotoxicity; can be induced by TNF.

Adapted from Clark et al.[14]

Bayoumi[5] proposes a relationship between genetic traits and the intensity and specificity of the protective immune response against *P falciparum*. The in vivo mechanism of protection against falciparum malaria in individuals of the Hb AS genotype is not due solely to the adverse influence of HbS on the intraerythrocytic development of *P falciparum*. Instead, the simple physiological effect of Hb S on parasite growth appears to trigger an in vivo process of enhancement of the intensity and/or specificity of the host immune response, leading to acquired protective immunity in a process simulating vaccination.

Immune responses and chemotherapy can overlap and complement each other in regard to oxidant stress. Antimalarial drugs may modulate the defensive function of human leukocytes by interfering with their oxidative metabolism. Chloroquine, quinine, mefloquine, and proguanil at high concentrations inhibit the oxidative burst whereas pyrimethamine, cycloguanil, tetracycline,[53] and primaquine[21] enhance it. The overall conclusion to be reached is that both vaccination procedures and drug design should take into account the effects on the oxidative burst.

ADAPTATION OF PLASMODIA TO OXIDANT STRESS

In the case of a thalassemic- or a G6PD-deficient erythrocyte, which is already susceptible to ROS, the invasion of such a cell by a parasite producing more oxygen radicals could prove suicidal. The parasite itself is not indifferent to the oxidant damage threatening its survival. One mode of adaptation is the preferen-

tially higher rate of invasion of young normal erythrocytes by malarial parasites, as compared with older cells within the same populations. Old, normal cells are less deformable than young ones. Likewise, the membrane of some cells containing abnormal hemoglobin exhibit a lower degree of fluidity. This could provide a unifying mechanism for decreased invasion of both old and abnormal erythrocytes.[81] and explain the preference for the young cells.

Roth et al[91] reported a reduction in parasitemias in G6PD-deficient cells. A retardation of *P falciparum* intracellular development in G6PD-deficient erythrocytes also has been shown.[34] Although it has been generally accepted that the parasite does not synthesize its own G6PD and uses the host protein, Shakespeare et al[97] suggest that *Plasmodium knowlesi* has its own G6PD. Luzzato et al[66] have demonstrated the presence of the parasitic gene for G6PD in *P falciparum*. Also, Hempelmann and Wilson[39] have reported an electrophoretically slow moving plasmodial protein with G6PD activity. Usanga and Luzzato[110] suggest that when the parasites develop in G6PD-deficient erythrocytes, they undergo adaptive changes to activate the G6PD gene and to produce their own enzyme which enables them to circumvent the unfavorable conditions presented by the abnormal host cell and to develop similarly in the deficient cell as in the normal one. Roth and Schulman[93] cultured *P falciparum* for five cycles in G6PD-deficient erythrocytes and challenged the parasitized erythrocytes with acetylphenylhydrazine, which served to exert an oxidative stress. Their results suggest that the adapted variant demonstrates only a moderate enhancement of resistance to oxidative stress in G6PD-deficient cells. The parasite adaptation may, therefore, be sufficient to compensate for only minor oxidative stress. There are variations in relation to the theory of adaptation: Lynn and Wilson[66a] established the presence of parasite-coded G6PD also in G6PD normal cells. In addition, we have recently found at least one variant of *P falciparum* (VNS) that cannot adapt itself to develop within the G6PD-deficient erythrocytes. It is important, therefore, to examine different variants of *P falciparum* in an area where malaria is endemic and where G6PD deficiency is ubiquitous to further evaluate the hypothesis presented by Usanga and Luzzatto.[110]

Plasmodia synthesize several histidine-rich proteins (HRP).[42,58,85,101] It is remarkable that each of the three known HRP genes of *P falciparum* can be deleted without loss of parasite viability, but simultaneous loss of all three HRP genes has not yet been observed.[52] The parasite has an intracellular route of transport for secretion of HRP, through several membranes and the host-cell cytoplasm.[42] Histidine analogs inhibit in vitro growth of *P falciparum*.[44] Thus, it is likely that HRPs play an important role in parasite survival. The knobs on the surface of parasitized erythrocytes contain mostly HRP and are responsible for the adherence to the endothelial cells. However, we propose additional possible roles for HRP. Because histidine and polyhistidine are scavengers of ROS,[12] and because histidine and polyhistidines bind heme and iron and consequently can lower the concentration of redox-available iron, both roles will limit the levels of deleterious free radicals that the parasite is exposed to. The results of Nnalue and Friedman,[74] showing that histidine can partially prevent parasite destruction by neutrophils, support this hypothesis.

Another aspect of the parasite defense is that plasmodia succeed in accumulating protective enzymes (ie, catalase, glutathione peroxidase, and SOD) beyond their concentrations in normal erythrocytes. *P falciparum* (FCR-3) contains an additional SOD, which unlike the erythrocytic enzyme is not cyanide-sensitive. However, parasites grown in erythrocytes that have been partially depleted of SOD are more sensitive to exogenously generated superoxide.[24]

DEVELOPMENT OF ANTIMALARIAL DRUGS

Vennerstrom and Eaton[111] suggest that in future designs of antimalarial drugs particular consideration should be given to the oxidant sensitivity of infected erythrocytes and to the effectiveness of oxidant drugs that act as antimalarials. In view of the growing problem of drug resistance and the present lack of an effective vaccine against any species of human malaria, the development of novel therapeutics (perhaps possessing oxidant effects directed specifically against the infected erythrocyte) should be vigorously pursued. In this extensive review[111] the investigators divide the antimalarials into two main groups: direct oxidants (the peroxides) and

the redox cycling xenobiotics that may catalyze the production of oxidants within the infected erythrocytes.

Additional new approaches to the development of novel therapeutics based on oxidant sensitivity have been investigated. The possibility of using polyamine analogs as targets for polyamine oxidases followed by release of ROS has been previously discussed.

The observation that favism occurs in a patient showing GR deficiency,[64] and not G6PD deficiency, suggests that the biochemistry of favism might be mimicked by inhibiting the flavoenzyme GR in human erythrocytes. This concept was supported experimentally by Zhang et al,[116,117] who showed that erythrocytes containing GR do not serve as host cells for *P falciparum*.

The three-dimensional structure of this enzyme is known,[79,96] so that the interactions with inhibitors can be studied in great detail,[50,51,78] which facilitates the design of new drugs with desirable properties (Fig 5). Reed[86] and Schirmer et al[95] have studied two GR inhibitors as antimalarials: the clinically used antineoplastic agent

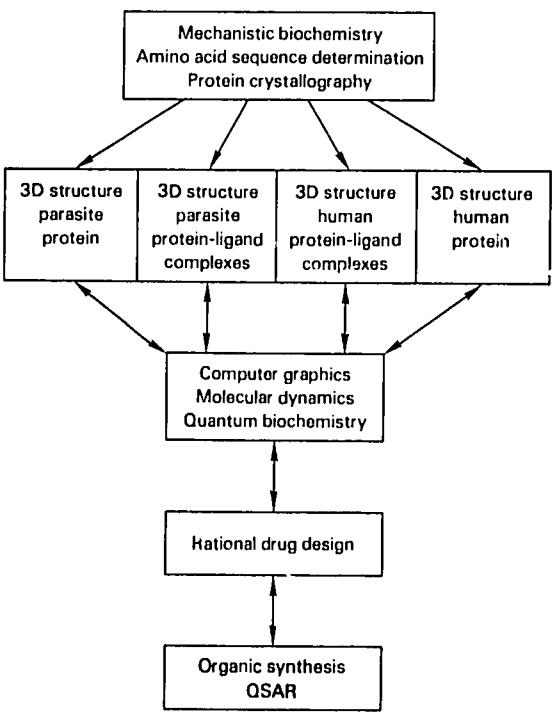

Fig 5. Flow diagram of successive steps in a drug design strategy where detailed knowledge of the structure of key proteins plays a central role. QSAR, quantitative structure-activity relationship. (From R.H. Schirmer, personal communication, 1988).

carmustine (BCNU), and its newly developed water-soluble and less toxic analog 1-(2-chlorethyl)-1-nitroso-3-(2-hydroxyethyl) urea (HeCNU). Both drugs inhibited the growth of *P falciparum* in culture. In addition, HeCNU was shown to have a curative effect on rodent malaria, being equally effective against chloroquine-resistant and chloroquine-sensitive strains of *P vinckei*.[96] Another inhibitor of GR, the riboflavin analog 10-(4'-chlorophenyl)-3-methylflavin, was found to have significant activity against *P vinckei* in vivo and against *P falciparum* in culture.[7,19]

Whenever GR is exposed to pharmacological inhibition its apoenzyme has a special significance. In regions where malaria is endemic, riboflavin deficiency is also common. As a consequence, a large proportion of GR molecules in erythrocytes lack their cofactor, flavin adenine dinucleotide (FAD). Erythrocytes with a higher proportion of apoenzyme will be less supportive for parasite development. Antimalarial chemotherapy, however, should aim both at the holoenzyme and at the apoenzyme, because the latter can be activated when riboflavin becomes available. On this account, FAD-free apoenzyme and holoenzyme were prepared, studied, and estimated in hemolysates with varying degrees of apoenzyme. Thus, the erythrocyte's glutathione reductase activation coefficient (EGRAC—a ratio of enzyme activity with or without the FAD cofactor) has a clinical importance. In the endemic area where malnutrition or thyroid malfunction exist, affecting riboflavin concentration in the erythrocytes, the EGRAC may reflect epidemiological factors that are part of the natural defense mechanism's against malaria and should be considered in the design of antimalarial drugs.[7]

Spira et al (personal communication, 1988) propose a novel approach to chemotherapy by using iron chelators. Their synthetic chelators may inhibit the malarial parasites by either iron deprivation or by the production of complexes that are redox-active and can support the formation of free radicals by the iron catalyzed Fenton reaction. Twenty μmol/L of 1-[*N*-ethoxycarbonylmethylpyridoxylidenium]-2-[2'-pyridyl] hydrazine bromide (L2-9) completely inhibited the in vitro development of *P falciparum*. Parasites at the trophozoite and schizont stages were more vulnerable to the chelator than the preceding

ring form, indicating again the higher sensitivity of the advanced stages to oxidant stress. Electron spin resonance rate signals produced by complexes of iron and L2-9 in the presence of spin traps indicate the production of free radicals. Stahel et al[100] found that iron chelators can also inhibit the liver exoerythrocytic forms of human malaria. Thus, iron chelators may be considered bona fide alternative candidates for existing antimalarial drugs.

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DELETERIOUS SYNERGISTIC EFFECTS OF ASCORBATE AND COPPER ON THE DEVELOPMENT OF *PLASMODIUM FALCIPARUM*: AN *IN VITRO* STUDY IN NORMAL AND IN G6PD-DEFICIENT ERYTHROCYTES

ESTHER MARVA,* ALONA COHEN,* PAUL SALTMAN,† MORDECHAI CHEVION‡§ and JACOB GOLENSER*

Departments of *Parasitology and ‡Cellular Biochemistry, The Hebrew University, P.O.B. 1172, Jerusalem 91010, Israel
† Department of Biology, University of California, San Diego, California, U.S.A.

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Abstract—MARVA E., COHEN A., SALTMAN P., CHEVION M. and GOLENSER J. 1989. Deleterious synergistic effects of ascorbate and copper on the development of *Plasmodium falciparum*: an *in vitro* study in normal and in G6PD-deficient erythrocytes. *International Journal for Parasitology* **19**: 779-785. The effects of ascorbate and copper on the development of *Plasmodium falciparum* were studied in two modes: *pretreatment* of uninfected erythrocytes followed by infection by *P. falciparum* and *treatment* of parasitized erythrocytes. *Pretreatment* of G6PD(+) cells with ascorbate caused a slight enhancement in parasite development, while in G6PD(−) cells a suppressive effect on the plasmodia was demonstrated. Copper alone interfered with parasite growth in both cell types. The combination of copper and ascorbate arrested parasite maturation, an effect which was more pronounced in G6PD(−) cells. Synergism between copper and ascorbate was better demonstrated following the *treatment* of infected erythrocytes: while ascorbate alone supported parasite development and copper alone had only a marginal suppressive effect, the combination of copper and ascorbate yielded a marked inhibition of parasite growth. Ascorbate proved destructive to the parasites in the presence of adventitious copper, or on the second day of the parasite life cycle. In these cases it acted as a pro-oxidant, while in other systems, in particular in the presence of a chelator, ascorbate acted as an antioxidant and promoted parasite growth. The understanding of the role of transition metals and free radicals in parasite development and injury could shed light on novel approaches to fight malaria.

INDEX KEY WORDS: *Plasmodium falciparum*; ascorbic acid; copper; free radicals; G6PD deficiency.

INTRODUCTION

OXIDATIVE challenge may affect the plasmodia at each of its developmental stages including recognition of host cells, penetration and intracellular development of the parasite (Golenser & Chevion, in press). For example, such a challenge may cause lipid peroxidation (Stocker, Hunt, Buffinton & Weidemann, 1985) or lead to the formation of methemoglobin (Wozencraft, Dockrell, Taverne, Targett & Playfair, 1984) that, in turn, may interfere with plasmodial growth. Likewise, *in vivo* studies using the mouse model have indicated that parasitemia is depressed by the oxidant stress-generating xenobiotic alloxan (Clark & Hunt, 1983). The effect of alloxan could be prevented by prior administration of the potent iron chelator desferrioxamine. These results are in full accord with the proposed catalytic role of transition metals in free radical-induced damage in a variety of biological systems (Chevion, 1988).

During the development of the parasite, hemoglobin is digested and a concurrent release of high levels of iron takes place within the red blood cell (Janney, Joist & Fitch, 1988). In this study, we have chosen to use adventitious copper as an exogenous metal in order to allow discrimination between the effects of the endogenous and added metals. Additionally, copper *per se* is known to act as an oxidant agent on red blood cells (Metz, 1960; Boulard, Blume & Beutler, 1975) even at concentrations compatible to its contamination in the environment (Calebrese, Gary & Soon-Ching, 1980).

The effects of copper should be better demonstrated in glucose-6-phosphate dehydrogenase (G6PD)-deficient erythrocytes which are more sensitive to oxidizing challenge (Friedman, 1979; Roth, Raventos-Suarez, Rinaldi & Nagel, 1983; Golenser, Miller, Spira, Navok & Chevion, 1983). In this investigation we have examined the effect of adventitious ascorbate, copper and a chelator (diethylenetriamine pentaacetic acid—Detapac) upon the development of *P. falciparum* in normal and in G6PD-deficient erythrocytes. It has been shown that ascorbate can act both as an antioxidant and as a pro-oxidant

§ To whom all correspondence should be addressed.

(Gutteridge & Wilkins, 1982; Hershko, in press). This will depend upon its concentration and the availability of redox-active transition metals, which catalyze its oxidation with concurrent production of free radicals and hydrogen peroxide (Gutteridge & Wilkins, 1982; Scarpa, Rigo, Orsega & Viglino, 1984). While the pro-oxidant effect is usually seen at low concentrations of ascorbate, at high concentrations the efficiency of ascorbate as a free radical scavenger highly exceeds its efficiency as a free radical producer and it becomes a potent anti-oxidant (Gutteridge & Wilkins, 1982).

MATERIALS AND METHODS

Experimental design. Two experimental approaches were examined: in the first, *pretreatment* of erythrocytes alone followed by infection with parasites was used, while in the second approach, *treatment of the parasitized erythrocytes* was employed. Experimental systems included *in vitro* cultures of *P. falciparum* in normal and G6PD-deficient erythrocytes. The parameters for parasite development were the uptake of radioactive hypoxanthine (Hx) and the level of parasitemia. Each of the experiments was repeated at least three times, and was performed in triplicate cultures. For each individual experiment the mean values (of the triplicates) and the deviation did not exceed 7.5%. In the course of our investigations, the parasites were cultured in blood from different donors. Additionally, an absolute standardization of the inoculum was not possible. These yielded results with different absolute values. However, the relative changes following pretreatments or treatments were the same. The figures and tables depict a typical set of results for each experiment.

Parasites. Plasmodium falciparum (strain FCR-TC), obtained from Professor J. Jensen, was cultured according to the method of Trager & Jensen (1976), with some modifications as earlier described (Golenser *et al.*, 1983), in 20 mM glucose. Cultures were synchronized by sorbitol treatment (Lambros & Vandenberg, 1979).

Blood. Blood was collected from normal healthy men or from G6PD-deficient (Med) individuals and stored at 4°C. The activity of G6PD in the deficient blood was less than 3% of the normal activity. Experiments were initiated within 72 h following collection of the blood.

Pretreatment. The blood was centrifuged, plasma and buffy coat were removed and the erythrocytes were washed twice with PBS (pH 7.2). Various concentrations of ascorbate, copper or combination of the two reagents were added in 2 ml of PBS to 0.1 ml of packed erythrocytes. The cell suspensions were incubated at 37°C for 1 h with gentle shaking, washed with RPMI-1640 medium to remove remaining reagents and suspended in growth medium. Suspensions enriched in trophozoites and schizonts (mature forms) were obtained by the gelation sedimentation technique (Jensen, 1978). An inoculum of parasitized erythrocytes was added to an erythrocyte suspension (5% hematocrit), so that the initial parasitemia was 1% and the contamination by untreated cells (originating from the inoculum) was less than 1.5% of the recipient cell population.

Treatment. Parasitized erythrocytes in their mature form were adjusted to 1% parasitemia in red blood cell suspension (5% hematocrit). Solutions of ascorbate, copper (as a sulfate), diethylenetriamine pentaacetic acid (Detapac) and their various combinations were prepared in PBS. Detapac was added first, at 37°C, 45 min later copper (freshly diluted from 100 mM solution of $CuSO_4$) followed by sodium ascorbate was added. The cells were dispensed into a 96-well microplate (100 μl per well) and incubated at 37°C using the candle jar method of Trager & Jensen (1976). The supernatant was replaced every day by medium containing the same reagents.

Parasite development. Incorporation of radioactive hypoxanthine (Hx) was measured according to the method of Golenser, Casuto & Pollack (1981) as follows: [^{3}H]-hypoxanthine (18.5 kBq per well, New England Nuclear) was added in 25 μl of medium to the culture. The cells were collected by filtration on glass microfibre filters and radioactivity was counted (by a Mimaxi β Tri-Carb 4000, Packard). The incorporation of Hx in normal erythrocytes alone (without parasites) did not exceed 1–3% of the lowest level of uptake by parasitized red blood cells. Parasitemia was measured daily using Giemsa-stained blood smears.

RESULTS

Pretreatment of uninfected erythrocytes

Normal [G6PD(+)] and G6PD-deficient [G6PD(−)] erythrocytes were pretreated with various concentrations of ascorbic acid or copper or their combinations, and subsequently infected with the parasites. There were minor effects of the pretreatment on Hx uptake in normal cells during the first day following treatment in culture, including a slight enhancement in Hx incorporation in the G6PD(+) erythrocytes pretreated with ascorbic acid (Fig. 1A).

The effect of copper alone on G6PD(−) cells was more prominent than on the G6PD(+) cells. Likewise, ascorbate alone exhibited increased effects in deficient cells, which were not shown in the normal ones. These enhanced effects in G6PD(−) erythrocytes were dose-dependent (Fig. 1B). A synergistic effect could be seen in deficient erythrocytes treated with 5 or 10 mM ascorbic acid and with 5 μM copper (Fig. 1B). The effects of ascorbic acid or copper were more pronounced on the second day following treatment (Figs. 1C and 1D). Then, the individual effects of either copper or ascorbate were too high to allow the demonstration of synergism.

Table 1 depicts the parasitemias (P) and the distribution of ring forms (R) and mature forms (M) after pretreatment (1 h exposure of non-infected erythrocytes to ascorbate and/or copper followed by inoculation of parasites; see Materials and Methods). On day 1, 19 h following *pretreatment* (Table 1a), no difference was recorded in parasitemias and in the distribution of the different developmental stages in G6PD(+) cells. Likewise, no difference was evident after the various pretreatments of G6PD(−) erythrocytes. The 7.5% parasitemia recorded at 10 mM ascorbate and 5 μM copper was the only value that was lower (in comparison with the results obtained without ascorbate or without copper).

Table 1b presents the parasitemia and stage distribution at 43 h following pretreatment of erythrocytes. The exposure of the G6PD(+) uninfected cells to ascorbate alone had resulted in a slight reduction of the parasitemias (3.5–4.2% vs 4.8% in the absence of ascorbate). However, most of the

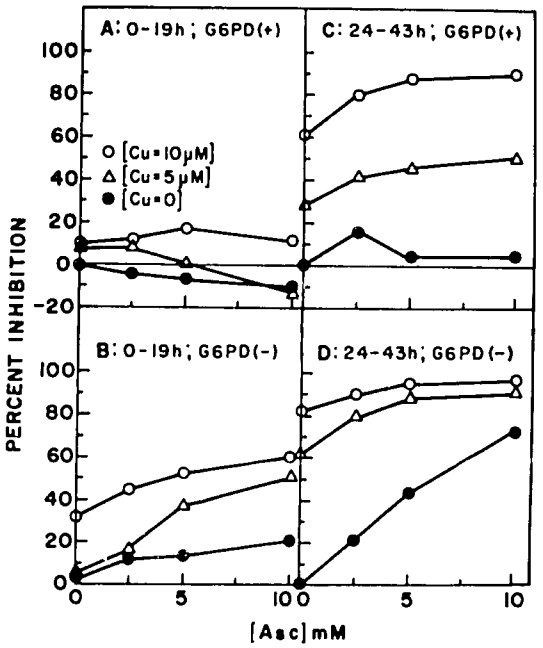

FIG. 1. The effect of ascorbate (Asc), copper and their combination on hypoxanthine incorporation after *pretreatment* of G6PD(+) or G6PD(−) non-infected erythrocytes, followed by inoculation with *P. falciparum*. The results are expressed as per cent inhibition of incorporation when compared to control cultures, pretreated with phosphate buffered saline. Panel A: The experiment was conducted in normal erythrocytes [G6PD(+)], and the incorporation was monitored for the first 19 h following pretreatment (0–19 h). Panel B: As in panel A, except for the parasites that were cultured in G6PD-deficient [G6PD(−)] erythrocytes. Panel C: As in panel A, except that the Hx incorporation was monitored for 19 h starting 24 h following pretreatment (24–43 h). Panel D: As in panel C, except for the parasites that were cultured in G6PD-deficient [G6PD(−)] erythrocytes.

TABLE 1a—THE EFFECTS OF ASCORBATE, COPPER AND THEIR COMBINATIONS ON PARASITEMIA AND STAGE DISTRIBUTION, 19 H AFTER PRETREATMENT OF NON-INFECTED ERYTHROCYTES, FOLLOWED BY INOCULATION WITH *P. falciparum*

Type of cell	Ascorbate (mM)	No $CuSO_4$			5 μM $CuSO_4$		
		P	R	M	P	R	M
G6PD(+)	0	7.0	98	2	7.8	100	0
	2.5	8.0	100	0	7.5	97	3
	5.0	8.3	100	0	8.0	98	2
	10.0	7.0	99	1	7.0	98	2
G6PD(−)	0	10.3	99	1	11.2	98	2
	2.5	11.2	99	1	9.2	100	0
	5.0	9.0	99	1	10.0	100	0
	10.0	10.0	100	0	7.5	97	3

P, Parasitemia (%).
R, Ring forms (% of young parasites).
M, Mature forms (% of trophozoites and schizonts).

TABLE 1b—THE EFFECTS OF ASCORBATE, COPPER AND THEIR COMBINATIONS ON PARASITEMIA AND STAGE DISTRIBUTION, 43 H AFTER PRETREATMENT OF NON-INFECTED ERYTHROCYTES, FOLLOWED BY INOCULATION WITH *P. falciparum*

Type of cell	Ascorbate (mM)	No $CuSO_4$			5 μM $CuSO_4$		
		P	R	M	P	R	M
G6PD(+)	0	4.8	0	100	3.6	9	91
	2.5	4.2	10	90	3.4	0	100
	5.0	3.5	15	85	3.4	5	95
	10.0	4.0	15	85	2.1	26	74
G6PD(−)	0	7.5	24	76	6.0	79	21
	2.5	7.2	25	75	6.8	75	25
	5.0	8.0	72	28	7.2	81	29
	10.0	6.0	79	21	5.5	80	20

P, Parasitemia (%).
R, Ring forms (% of young parasites).
M, Mature forms (% of trophozoites and schizonts).

in the absence of ascorbate). However, most of the surviving parasites had continued to develop from the ring to the mature forms. Pretreatment of G6PD(+) erythrocytes with copper alone resulted in a similar effect. The combination of copper (5 μM) and ascorbate (10 mM) caused the highest degree of parasite killing and also interfered with the development of the parasites to the mature form, 26% of the surviving parasites remained as ring forms.

Exposure of G6PD(−) red blood cells to the higher concentrations of ascorbate alone (5 or 10 μM) had not affected the degree of parasitemia, when compared to control, but had resulted in a suppression of the development to the mature form, and most of the parasites remained in the ring form. Likewise, copper alone or the combination of copper and ascorbate (2.5 or 5 mM) induced the same phenomenon: no change in parasitemia with high incidence of ring forms. The combination of 10 mM ascorbate and 5 μM copper resulted in a slightly higher inactivation, which was more obvious on the third day (not shown).

Treatment of parasitized erythrocytes

G6PD(+) parasitized cells were exposed to ascorbate, copper or their combination and their effects were evaluated by monitoring the level of parasitemia and the distribution between ring and mature forms, and by the incorporation of Hx. In the first day ascorbate alone enhanced Hx incorporation (Fig. 2A) and increased the level of parasitemia (Table 2). This was probably associated with increased efficiency of the initial parasite invasion in the presence of ascorbate alone.

On the second day (24–43 h), when most of the parasites had already developed to the mature form, ascorbate alone caused a significant suppressive effect. For example, 3 mM ascorbate reduced the parasitemia from 10.3 to 7.2%; this probably represented the corresponding destruction of parasites. Concomit-

antly, an inhibition in Hx incorporation was exhibited by ascorbate alone (Fig. 2B). This ascorbate effect was even more pronounced in the next life cycle of the parasites (48–67 h, data not shown).

Copper alone exerted a suppressive effect, on the second day of the experiment, which was exhibited both by the parasitemias and the incorporation of Hx (Figs. 2–3, Table 2).

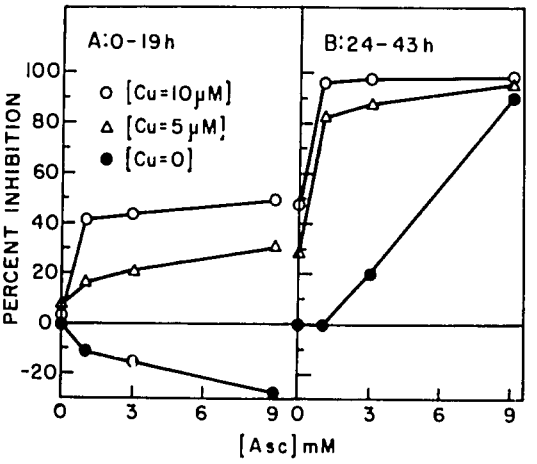

FIG. 2. The effect of ascorbate (Asc), copper and their combination on hypoxanthine incorporation following *treatment* of *P. falciparum*-infected erythrocytes. The results are expressed as per cent inhibition of incorporation when compared to control cultures, treated with phosphate buffered saline. Panel A: The incorporation of Hx was monitored for 19 h following treatment (0–19 h). Panel B: The incorporation of Hx was monitored for 19 h starting 24 h following treatment (24–43 h).

Exposure of infected cells to the combination of ascorbate and copper resulted in a synergistic suppressive effect indicated by both the parasitemia and by the incorporation of Hx on the first day (0–19 h Fig. 2 and Table 2). While ascorbate alone (3 mM) caused a 15% *increase* in Hx incorporation (−15% inhibition) and copper alone (10 μM) had only a minor effect (3% inhibition), their combination resulted in a 43% inhibition. The marked decrease in both parameters was more pronounced on the second day of the experiment. Exposure to the combination of copper and ascorbate resulted in a complete arrest of parasite development, so that most parasites remained in the ring form (>90%). This was associated with excessive degradation of infected cells, as reflected by a reduced level of parasitemia. During both days, the pattern of parasite stage distribution in treated infected cells was similar for the controls and those cells treated with either copper alone or ascorbate alone.

The effect of the chelator Detapac

The role of transition metals in the induction of oxidant stress was further examined by the addition of the chelator diethylenetriamine pentaacetic acid (Detapac). Detapac was added to the parasitized erythrocytes before the addition of ascorbate or copper, and remained in culture throughout the experiment. The addition of Detapac (100 and 200 μM) to parasitized erythrocytes caused a 12% increase in Hx incorporation on the first day. Detapac completely reversed the inhibitory effect induced by copper alone and by the combinations of copper and ascorbate (Figs. 3A–C). In addition, Detapac enhanced Hx incorporation in ascorbate-treated cells. A similar effect was demonstrated on the second day (Figs. 3D–

TABLE 2.—PARASITEMIA AND STAGE DISTRIBUTION OF PARASITIZED ERYTHROCYTES TREATED WITH ASCORBATE, COPPER OR THEIR COMBINATIONS IN THE PRESENCE OR ABSENCE OF DETAPAC

Detapac (μM)	Time post treatment (h)	Ascorbate (mM)	No $CuSO_4$			5 μM $CuSO_4$		
			P	R	M	P	R	M
0	19	0	8.0	99	1	7.0	99	1
		1	12.5	99	1	4.4	100	0
		3	12.3	100	0	3.0	100	0
	43	0	10.3	12	88	7.5	20	80
		1	9.5	10	90	5.2	95	5
		3	7.2	4	96	2.5	97	3
200	19	0	8.0	98	2	6.1	100	0
		1	11.0	99	1	6.0	100	0
		3	10.5	99	1	6.4	100	0
	43	0	8.0	8	92	6.5	8	92
		1	10.4	10	90	7.4	6	94
		3	6.8	6	94	5.7	3	97

P, Parasitemia (%).
R, Ring forms (% of young parasites).
M, Mature forms (% of trophozoites and schizonts).

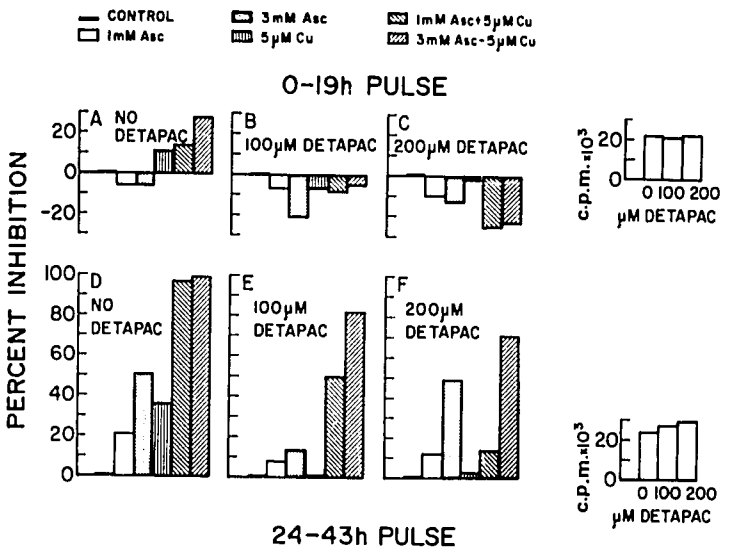

FIG. 3. The effect of diethylenetriamine pentaacetic acid on hypoxanthine incorporation following *treatment* of *P. falciparum*-infected erythrocytes. Treatment with copper, ascorbate (Asc) and their combination was conducted as described in Materials and Methods, and shown in the figure. The results are expressed as per cent inhibition of incorporation as compared to control cultures. The effect of Detapac alone is shown (in c.p.m.) on the right. Panels A–C: Hx incorporation was monitored in infected erythrocytes for 19 h (0–19 h) following the onset of the experiment. Panels D–F: Hx incorporation was monitored in infected erythrocytes for 19 h, starting 24 h after the onset of the experiment (24–43 h).

F). Then, the effects of ascorbate, copper and their combinations were more pronounced. However, Detapac induced a marked reduction of all the inhibitory effects exerted by either agent. Furthermore, Table 2 presents the parasitemias and parasite-stage distribution in the presence of adventitious Detapac. It shows that this chelator caused the partial reversal of the inhibition induced by ascorbate and copper. Comparison of the effect of ascorbate (3 mM) to that of ascorbate (3 mM) and copper (5 μM) shows a decrease in parasitemia from 12.3 to 3% (Table 2, 19 h), while in the presence of Detapac, an analogous comparison shows a smaller decrease from 10.5 to 6.4% (Table 2, 19 h). Similarly, without the chelator, the addition of 3 mM ascorbate to 5 μM copper reduced parasitemia from 7.5 to 2.5% (Table 2, 43 h). In the presence of Detapac, the same concentrations reduced the parasitemia from 6.5 to 5.7% (Table 2, 43 h). The effect of Detapac on stage distribution is also shown in Table 2: after 43 h in culture, parasites treated with copper and ascorbate failed to develop from ring to mature forms. In contrast, in the presence of Detapac this interference with parasite development was relieved and about 95% of the parasites developed into mature stages.

DISCUSSION

Oxidative stress has already been incriminated as a deleterious factor in the development of malaria parasites (Friedman, 1979; Clark & Hunt, 1983; Stocker *et al.*, 1985; Allison & Eugui, 1982). However, the mechanism by which the parasite is affected by such a challenge needs further clarification. Various chemical reductones (Schank, 1972; Chevion, Navok, Glaser & Mager, 1982) which can undergo cyclic oxidation and reduction, such as dialuric acid/alloxan (Clark & Hunt, 1983), isouramil (Golenser *et al.*, 1983), divicine (Clark, Hunt, Cowden, Maxwell & Mackie, 1984) or butyl hydroperoxide (Clark, Mackie & Cowden, 1986), have been shown to cause oxidative stress in red blood cells. Likewise, ascorbate, a naturally-occurring redox-active compound, can induce the formation of active oxygen-derived species, such as superoxide radicals ($O_2^{\cdot}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\cdot}$). It is widely accepted that the hydroxyl radical could be the ultimate deleterious species (Halliwell & Gutteridge, 1984). Also, it is generally assumed that its formation is mediated by the redox-active and available transition metals iron and copper (Chevion, 1988; Aust, Morehouse & Thomas, 1985).

The effects of oxidative stress induced by ascorbic acid and copper in our studies of *P. falciparum* have focused on three parameters: (1) the role of exogenous redox metals, in particular copper, (2) the ability of added chelator, diethylenetriamine pentaacetic acid (Detapac), to distinguish between exogenous and endogenous metals, and (3) the comparison of the effects in both G6PD(+) and G6PD(−) cells.

Pretreatment of G6PD(+) cells with ascorbate caused only a small effect on the development of the parasite. There was even a slight enhancement in Hx incorporation in these pretreated cells on the first day (Fig. 1). In contrast, in G6PD(−) cells, ascorbate

exhibited significant suppressive effects (Fig. 1), which did not affect merozoite invasion, but was reflected by suppression of the intracellular development of the ring forms to mature forms (Table 1b). The enhanced effects in G6PD(−), which are known to be more susceptible to oxidative stress, provide additional evidence that the deleterious effects of copper/ascorbate are mediated by active oxygen-derived species.

Infected G6PD(+) erythrocytes exposed to treatment with copper and/or ascorbate demonstrated a synergism in both Hx incorporation and parasitemia (Fig. 2, Table 2).

On the first day following treatment, ascorbate alone increased Hx incorporation and enhanced the level of parasitemia. This was probably associated with the increased efficiency of parasite invasion. It is possible that at this stage of parasite development the ascorbate served as an antioxidant. It seems to correlate with the earlier findings by Stocker, Hunt, Weidemann & Clark (1986) who found elevated levels of vitamin C in parasitized erythrocytes from mice infected with *P. vinckei*. They suggested that the content and redox state of erythrocytic ascorbic acid can be altered to protect the parasitized red cell and its contents from oxidative damage.

On the second day, when most of the parasites had developed to the mature form, ascorbate alone had already been exerting a suppressive effect, and the parasitemia dropped by 30%. Concomitantly, a corresponding inhibition in Hx incorporation was exhibited by ascorbate alone. On the first day, the beneficial effect of ascorbate for the parasite was probably due to its antioxidant characteristics. On the second day, when the parasites had reached maturity, and most of the hemoglobin had already been digested and the heme released, the effect of ascorbate could be attributed to its increased iron-mediated pro-oxidant potency.

The role of transition metals in the enhancement of oxidant stress was further examined by the addition of the chelator Detapac to the experimental system. The addition of Detapac (100 and 200 μM) to parasitized erythrocytes completely reversed the inhibitory effect of ascorbate alone, copper alone and their combination on the first day. The effect of adventitious copper was completely eliminated by Detapac on the second day as well. Detapac which is a chelator that forms tight complexes with iron and copper (Pessin, 1979) and does not penetrate into cells (Hershko, Grady & Cerami, 1978) could not prevent the ascorbate-induced inhibition of Hx-incorporation on the second day. This inhibitory effect of ascorbate is probably mediated by the newly released yet undefined form of intracellular iron. The effect of Detapac is in accord with the protection provided by desferrioxamine in alloxan-induced damage to plasmodia in mice *in vivo* (Clark & Hunt, 1983). It could also be reconciled with long-term exposure to desferrioxamine (Fritsch & Jung, 1986). There, the chelator deprives the parasite from utilizing iron, which is essential for the parasite growth (Raventos-Suarez, Pollack & Nagel, 1982; Oppenheimer, Gibson, MacFarlane, Moody, Harrison, Spencer & Bunari, 1986), and by this enhances the damage rather than provides the expected protection.

The results in this study suggest that the deleterious effects of ascorbate are metal-dependent and occur via a free radical pathway. The synergism between ascorbate and copper substantiates this suggestion. Our understanding of the role of transition metals, reducing agents and free radicals in parasite development and injury provides novel strategies in the fight against malaria.

Acknowledgements—This study was supported by grants from the Council for Tobacco Research (U.S.A.) (No. 1900), from AID/CDR (No. C7-163), by research contract No. 1-A1 22668-NIH-NIAID, and U.S. N.S.F. (CHG 8620149). P.S. was Lady Davis Visiting Professor at the Hebrew University.

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Report on the visit of Professor M. Chevion of the Department of Cellular Biochemistry and Prof. J. Golenser of the Kuvin Centre for Tropical Diseases, The Hebrew University, Hadassah Medical School, Jerusalem - ISRAEL.

Date: March 29-April 12.

This visit was made so the two groups in the collaborative project "Protective mechanisms against Malaria associated with G-6PD deficiency" could meet, hold discussions on the topic and seek further ways of expanding the project. It was very important that the ISRAELI team meet their Cameroon counterparts and see the potential and feasibility for such work to be carried out in Yaoundé.

During their stay in Yaoundé, they met with various Politicians, administrative persons, representatives of International Organizations and Scientists, a list of which is attached. They also spent a day out of Yaoundé to get a feeling of what the rural area is like, where field work will be taking place. They visited Obala and Batchenga.

Four lectures and an informal discussion were given, as seen in the programme. Discussions on the project with the collaborating Scientists went on very well. As regards Malaria Parasites (P. falciparum) that have been established in culture will be sent to Yaoundé from ISRAEL and the Scientists in Yaoundé would try to get the cultures going. Procedures for cultivating the parasites, for synchronisation, for verification of strain differences, were discussed.

The discussions followed the Outline for proposed experiments in Cameroon.

1) Facilities for culturing Plasmodium falciparum will be established in Yaoundé. Techniques for synchronization and measurements of parasites development will be adapted.

2) Various strains of P. falciparum will be isolated from patients with active malaria. These strains will be characterized by their isoenzymes and reaction with specific monoclonal antibodies.

3) Blood from human beings will be monitored for glucose-6-phosphate dehydrogenase activity. If deficiency is found an attempt will be made to use this blood from relatives, to support the growth in culture of the parasite.

4) Some isolates of P. falciparum will be grown in bloods which are normal or deficient with respect to G6PD.

5) The effect of selected favism inducing agents (Fava bean extracts and other oxidants will be examined on specific combination of P. falciparum isolated in Cameroon, and grown there, in erythrocytes with varying degrees of G6PD activity.

6) The effects of reactive oxygen species produced by the immune system will be examined with activated cells (or their products). These cells will be obtained from donors unexposed to malaria, or infected with malaria, or immune.

It was decided that our local foods (fruits, vegetables) be verified for products which cause oxidant stress. Apollinaire when going to ISRAEL will carry with him local fruits and vegetables, and also extracts (or pure plants) of known medicinal plants (see Prof. TITANJI if he be interested to send some of his anti-filarial Cpds). These will be tested for oxidant stress indicators.

A field study was proposed: to study the effect of ascorbate on the prevalence of malaria in defined areas, following results of in vitro studies using Ascorbate. This project will be carried out shortly in an animal model in Jerusalem, and the plans will be made for the field trials around Yaoundé. Funds will have to be obtained for this, and as an addition to the existing project.

As regards funding, both teams visited some funding Agencies and discussed the possibilities, requirements and funding procedures. These were: USAID, FAO and UNDP. A proposal should go to USAID within the next ten days (10 days).

Some materials and reagents for the project will preferably be paid for from ISRAEL and have them shipped to Yaoundé. A list is enclosed, and it is understood that the ISRAELI team will do as much as they can, and what is not possible will be left out.

Scientists from both teams will visit the other laboratory, depending on availability of funds. it was suggested that Drs. LEKE and NKUO should make their 1st visit to ISRAEL and plan to spend about 3 months, and do a little project during that time. Funds will be requested for this in proposals to funding agencies.

Visit of Professor M. Chevion of the Department of Cellular Biochemistry and Prof. J. Golenser of the Kuvin Centre for Tropical Diseases, The Hebrew University, Hadassah Medical School, Jerusalem.

March 29		
Wednesday	10H00	Visit C.U.S.S. Immunology Labs and Scientists Meet Director of C.U.S.S.
	15H30	Meet Vice-Chancellor Meet Chancellor
March 30		
Thursday	8H30-12H00	Discussions on Collaborative Project in CHU Laboratory
	15H00-17H00	Discussions continue
	18H00	Lecture: The Involvement of free radicals and Transition Metals in the toxicity of pyridinium compounds. By Prof M. Chevion. Faculty of Science, Amphi: A1 or A2
March 31		
Friday	8H30-12H00	Visit Biotechnology Centre Visit OCEAC
	15H00-17H00 EVENING	OPEN Private Dinner with Vice-Chancellor
April 3		
Monday	8H30	Meeting with Minister of Higher Education Visit CHU
	15H00	Discussions
	18H00	Lecture: The Involvement of free radicals and transition metals in ischemia and reperfusion-associated injury. By Prof M. Chevion. Faculty of Science Amphi: A1 or
April 4 Tuesday	8H30-12H00	Meeting with Minister of Health Visit Hôpital Central
	15H00-18H00	OPEN
April 5		
Wednesday	8H30-12H00	Discussions
	15H00-17H00	Discussions
	17H30	Lecture: Destructive effect of Oxygen free radicals on malaria parasites. By Prof J. Golenser. C.U.S.S.

April 6 Thursday	8H30-12H00	Visit Schistosomiasis Project-Nkomo Visit Director IMPM - 9.30AM.
	15H00-17H00	Discussions
	18H00	Lecture: Mechanisms of destruction of Malaria parasites by free radicals. By Prof. J. Golenser. C.U.S.S.
April 7 Friday		USAID, FAO, PNUD Discussions Final day with Prof M. Chevion
April 8 Saturday		Departure of Prof. M..Chevion
April 10 Monday		Discussions with Prof. Golenser
	17H30	Talk by Prof. Golenser on: The Scientific activities of the Kuvin Centre for Tropical Diseases
April 11 Tuesday		OPEN
April 12 Wednesday		Departure of Prof. Golenser

LIST OF PERSONS MET

Prof. Pierre Carteret :	Director of CUSS
Prof. Same Ekobo :	Professor of Parasitology in CUSS
Dr Mbuntum :	Secretary General, CUSS
Prof. Mounan :	Visiting Prof. from University of Alberta

Dr ASONGANYI Dr LANDO Dr Peter NDUMBE	WHO-Collaborating Centre: Research on Immunology of Onchocerciasis, Trypanosomiasis, malaria, etc.

Dr jeanne Ngongang: Biochemistry CUSS.

Prof. Vincent Titanji	Dept of Biochemistry, Fac of Science plus WHO Collaborating Centre plus Scientific Secretary, Biotechnology Centre

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Mr Abolo	Administrative Secretary Biotechnology Centre
Dr Tsala	Ecole Normale Supérieure Scientific Secretary: Biotechnology Centre (plant)
OCEAC:	Secretary General
Dr Pierre Carnevale	
Dr Gazin	
Dr HENGY	
Dr Bert Mulder (from Neimegen)	

Minister of Higher Education: Honorable Minister BABALE

Prof. Kamdom-Moyou	Director of CHU Hospital
Dr Leopold Zekeng	Hematologist, CHU
Gary Leinen	USAID
FAO	
UNDP	
Prof. ANTOINE ABONDO	Director IMPM
Dr Jonas KUAMO	Biochemist: Parasitology Lab. Institute of Medical Research (IMPM) Working on choloroquine resistance
Dr Lysette Kouemeni:	IMPM/USAID Tulane Schistosomiasis Project
Dr MIMFOUNDI	Malacologist "
Dr George Greer	Malacologist (Tulane) "

LIST OF MATERIALS AND REAGENTS

Parasites

Cutoclavable filters

6-well culture dishes

Powdered RPM1

Sorbitol

Gelatine

Sterile plastic tubes

Sterile pipettes

Drug Sensivity plates: Reickman

Slides for IFAT

Candles

Clamps

Centrifuge

Small Water Distiller

Q: Is there a possibility to get a decent T° Control Centrifuge for the amount allocated for equipment for Cameroon?

Please send: .. Protocols for culture, synchronisation, strain identification

.. Articles on:

- Maternal transfussion of antibodies to foetus
- Immunology of Schistosomiasis : Review
- RESA : pf 155 : Any new papers
- Malaria immunology : Relevant articles : on CSP and asexual blood stage ages.