PROTECTIVE MECHANISMS AGAINST MALARIA ASSOCIATED WITH GLUCOSE 6-PHOSPHATE DEHYDROGENASE (G6PD) DEFICIENCY

Final Scientific Report

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Supported by
United States Agency for International Development
US-Israel CDR Programme C7-163 Grant DPE-5544-G-SS-8039-00

April 1993

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Introduction

Malaria, a disease caused by various species of \textit{Plasmodium}, mostly by \textit{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 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 erythrocyte glucose-6-phosphate dehydrogenase (G6PD) deficiencies, heterozygosity for hemoglobin S, thalassemias, heredity persistence of fetal hemoglobin and hemoglobin E. All of the above erythrocytes are particularly sensitive to oxidant stress and to parasite-induced oxidation.

Malarial plasmodia constitute a typical example of intracellular parasitism. As with all host cell-parasite interactions, this consists 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.

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 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 other 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.

We thereby report the work which was done within the framework of the AID/CDR, particularly: (a) genetic aspects of the *plasmodium*, (b) oxidant stress and malaria -- mechanisms responsible for the destruction of malaria parasites, and (c) single electron redox reactions of chloroquine, primaquine and quinine, which may explain their antimalarial activity.
The results were included in some publications. A list of the references resulting from this effort is enclosed.

Genetic Aspects of P. falciparum: Enzyme Typing of African Isolates by Polyacrylamide gel electrophoresis

Diversity within different species of parasites is a major reason why parasites survive despite of the ability of their hosts to mount immune responses which are effective in eliminating a particular infecting population. Diversity in P. falciparum is seen in a variety of characteristics which include antigens, levels of drug resistance, morphology and karyotype, in addition to isoenzymes patterns which will be discussed here. Significant antigenic diversity in malaria parasites of humans was demonstrated when individuals deliberately infected with malaria were found to be relatively more resistant to subsequent infection with the homologous strain than with a heterologous strain (Jeffery, 1966). The slow development of immunity in people living in areas where malaria is endemic is consistent with the hypothesis that immunity only develops after exposure to a large number of different parasite strains.

In recent years there has been great progress in characterizing antigens of the different life-cycle stages of P. falciparum and the molecular basis for much of the antigenic
diversity among different parasite populations is now known. In this report we summarize our effort of enzyme typing of the African isolates of *P. falciparum*.

Isoenzyme electrophoresis has proved to be a useful tool for determining genetic variation within and among populations of *P. falciparum* (Beale & Walliker, 1988). Several studies have shown that similar forms of enzymes occur in populations from different geographical regions, suggesting that *P. falciparum* exists as a single interbreeding worldwide population. However, the frequencies of variants have been found to differ among regions. Furthermore, some isolates have been shown to possess more than one form of a given enzyme. These are assumed to be clonal mixtures of parasites having different forms of the enzyme.

Most investigations of isoenzyme variation in *P. falciparum* have been carried out using starch gel electrophoresis. However, we found that resolution of bands was improved by using polyacrylamide gels. The results of a study of the variation in 5 enzymes of 19 culture-adapted African isolates of *P. falciparum* are reported.

**Materials and Methods**

*P. falciparum* isolates were obtained from patients who became infected in Mozambique, Malawi and South Africa, all being endemic malarial areas. The Gambian isolate, FCR-3 (Jensen
& Trager, 1978), which was characterized by Sanderson et al. (1981), was used as a reference sample.

The isolates were grown in gassed tissue culture flasks to a parasitemia of at least 1% schizonts. Parasites were released from their host erythrocytes by saponin lysis as described by Sanderson et al. (1981), and further lysed using 1% Triton X-100 in ethylenediaminetetraacetic acid-Tris-HCl buffer pH 7.4 (Thaithong et al., 1981). Material for electrophoresis was prepared from each isolate on 3 separate occasions during 11 months of continuous cultivation. Lysates were stored in liquid nitrogen until required.

Electrophoresis was carried out on 7.5% polyacrylamide gels with \( \approx 2.8\% \) cross-linkage. The enzymes studied were glucose phosphatase isomerase (GPI) (EC 5.3.1.9), adenosine deaminase (ADA) (EC 3.5.4.4), lactate dehydrogenase (LDH) (EC 1.1.1.27), NADP-dependent glutamate dehydrogenase (GDH) (EC 1.4.1.4), and 6-phosphogluconate dehydrogenase (PGD) (EC 1.1.1.44). The conditions of electrophoresis for GPI, LDH and GDH were basically as described by Carter (1978), and for PGD and ADA as described by Harris & Hopkinson (1976).

Results and Discussion

There was little variation amongst the African isolates and all enzymes showed similar forms in isolates from the different geographical areas. Ten of the 19 isolates exhibited the same electrophoretic pattern as the Gambian isolate, FCR-3. GDH and
PGD revealed no variation amongst the isolates. This has been the experience of other workers (Beale & Walliker, 1988). GPI exhibited the most variation, while ADA and LDH showed limited variation.

The enzyme frequencies of the African isolates were similar to those of isolates from other parts of the world (Beale & Walliker, 1988). GPI-1 occurred in 11 of the 19 isolates tested, while the remaining 8 had both GPI-1 and GPI-2. The ADA frequencies were similar to those found in the Gambia and Thailand, where over 90% were ADA-1. Eighteen of the isolates studied had ADA-1, while the remaining isolate had both ADA-1 and ADA-2. LDH-2, which has been found only in African isolates, appeared with LDH-1 in one isolate.

Of the 8 isolates possessing both GPI-1 and GPI-2, only one retained both variants throughout the test period. In 4 of these isolates, GPI-2 was seen only in the first lysate but not in lysates prepared subsequently. A further 3 isolates exhibited only GPI-1 in the first and second tests but both GPI-1 and GPI-2 in the third test. Similarly, one isolate had both ADA-1 and ADA-2 in the first and third lysates, but only ADA-2 in the second lysate. The ADA-2 band in the first and third lysates was much darker than the ADA-1 band, thus indicating a higher proportion of parasites with ADA-2.

These changes in the electrophoretic pattern of an isolate with time may be ascribed to the relative numbers of parasites possessing the different forms of the enzymes at the time of
preparation of lysates. Thus, parasites exhibiting a variant which appears after some time in culture may have grown from an undetectable number of organisms to a number sufficient to produce a band on the stained gel. Parasites characterized by a variant which disappears after some time in culture may be present in undetectable numbers or may even have died out in culture.

The results of this study lend further support to the conclusion that *P. falciparum* isolates from different geographical regions constitute a single species.

**Genetic Aspects of P. falciparum:**

**Typing of African Isolates Using Monoclonal Antibodies**

The ever-increasing problem of drug resistance shown by malarial parasites has given the development of new drugs 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 serotyped 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 have resulted in the loss of organisms specific for
monoclonal antibodies 9.2 and 10.3 in culture. Four isolates possessed 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*. 
Modified bases occur, with a very few exceptions, in the DNA of all prokaryotes and eukaryotes (Razin & Friedman, 1981). In *Escherichia coli* DNA, both 6-methyladenine and 5-methylcytosine are found (Doskocil & Sormova, 1965) in GATC and CCATGG sequences, respectively (Razin *et al.*, 1980). Methylated adenine residues were also observed in several lower eukaryotic protozoans (Ray & Steele, 1978). In higher eukaryotes, 5-methylcytosine is the only methylated base (Wyatte, 1951) and is present only in the dinucleotide CpG (Sinhimer, 1955). There is ample evidence suggesting that methylation plays a significant role in gene expression (Razin *et al.*, 1984; Ceder, 1988). Very little is known about methylation of the plasmodial genome. Previously, we reported the lack of both 6-methyladenine and 5-methylcytosine (Polleck *et al.*, 1982). This conclusion was based on HPLC (high performance liquid chromatography) analysis, as at that time, specific DNA probes were not available. Taking into consideration the extremely low level of the G+C content of the parasite's DNA (18%) (Pollack *et al.*, 1982), and the fact that there is a 50% underrepresentation of the dinucleotide CpG in the genome (Hyde & Sims, 1987; Weber 1987), HPLC may not be sensitive enough to detect low levels of methylation. Here we describe the results of a restriction enzyme analysis of the
methylation status of cytosine and adenine in the DHFR-TS gene of *P. falciparum*.

Phylogenetically *P. falciparum* is classified as a lower eukaryote. However, comparison of the amino acid sequence of several plasmodial proteins with that of their functionally equivalent proteins from other organisms revealed a higher degree of homology with the vertebrate sequences rather than with those from lower eukaryotes (Hyde & Sims 1987; Bzik *et al.*, 1987; Bianco *et al.*, 1986; Yang *et al.*, 1987; Ardeshir *et al.*, 1987). Another property of the plasmodial genome, which is typical to vertebrate genomes, is the underrepresentation of the dinucleotide CpG (Hyde & Sims, 1987; Weber, 1987). This fact may be indicative of the presence of 5-methylcytosine in the plasmodial genome as it was shown that in eukaryotes, there is a direct correlation between the degree of CpG underrepresentation and the extent of cytosine methylation of this sequence; the higher the degree of underrepresentation, the higher the level of methylation (Bird, 1980). Previous attempts to detect 5-methylcytosine using either a chemical procedure (Pollack *et al.*, 1982) or a comparative MspI/HpaII restriction analysis of four plasmodial genes (Weber, 1987), failed to show it. Although these procedures are reliable, they cannot be conclusive when applied to genomes extremely low in their G+C content and having very low CpG-containing restriction sites for enzymes which are used for that purpose. For example, the circumsporozoite gene, whose methylation status was studied
using *MspI/HpaII* analysis (Weber, 1987) does not contain any such site in its coding region (Dame et al., 1984). Neither are there any *MspI/HpaII* restriction sites in the DHFR-TS gene (Bzik et al., 1987; Cowman et al., 1988). It is therefore obvious that *MspI/HpaII* analysis cannot be used to exclude definitely the existence of methylated cytosine residues in the genome of *P. falciparum* and that other enzymes whose activity is sensitive to methylation should be used. In this study, in addition to *MspI/HpaII*, we used several such enzymes. Using the enzymes *Sau3AI* and *MboI*, we were able to detect in all the isolates tested, a CpG site in the DHFR-TS gene which was partially methylated.

Restriction enzymes are the most commonly used tool for the detection of methylated bases. Some caution should be taken, however, when interpreting the results, as it has been shown, in a small number of cases, that a restriction enzyme may exhibit a lower affinity than normal toward a certain recognition site in the DNA molecule (Brown & Smith, 1977; Forsblum & Philipson, 1976; Gingeras & Brooks, 1983; Thomas & David, 1975). This may lead to a slower rate of cleavage of this site, or, in extreme cases, to the lack of its cleavage. However, neither one is the case in this study as (a) the 918-bp fragment behaves kinetically in a manner similar to that of the other fragments; (b) the GATC site, whose methylation results in the appearance of this fragment, is cleaved by *Sau3AI*. 
In conclusion, evidence is presented for the existence of 5-methylcytosine in *P. falciparum* DNA. The methylation is of the eukaryotic type by virtue of its appearance in a CpG site and by the fact that it was partial.

**Malaria Derived Redox-Active Containing Structures**

**Play an Important Role in Inducing Oxidant Stress:**

**Hemin Is a Possible Candidate**

Malaria parasites are very sensitive to the toxic effects of reactive oxygen species (ROS), such as superoxide (·O₂), hydrogen peroxide (H₂O₂) and hydroxyl radicals (OH’), which arise spontaneously within parasitized erythrocytes (Hebbel *et al.*, 1982; Marva *et al.*, 1990). These activated oxygen species may be generated by various chemical reductones (Schank, 1972; Chevion *et al.*, 1982), which can undergo cyclic oxidation and reduction, such as dialuric acid/alloxan (Clark & Hunt, 1983), isouramil (Golenser *et al.*, 1983), divicine (Clark *et al.*, 1984) or ascorbate (Marva *et al.*, 1989). Additionally, all of the above have been shown to cause oxidative damage to infected red blood cells. It is widely accepted that the hydroxyl radical species can cause deleterious damage (Halliwell & Gutteridge, 1984), and that its formation in various biological systems is mediated by the re:ox-active available transition metals, iron and co-factor (Chevion, 1988).
not add to DNA, the hydroxyl radical does (Ames, 1984). The hydroxyl radical is the only oxyradical that has a strong propensity to undergo H-abstraction reactions from both deoxyribose moiety (leading to single strand nicks) and the bases of DNA (converting thymine to hydroxymethyl uracil) and additional reactions, and is the most electrophoretic one. The hydroxyl radical is so reactive that it can only diffuse 5-10 molecular diameters before it reacts, so if it is not produced near the DNA it probably will not react with it.

DNA is not cleaved efficiently to the non-redox metal induced mechanism (Basile, 1987). However, DNA degradation by radicals formed in metal catalyzed autooxidation of ascorbate (or other reducing agent) is well known. This metal-catalyzed process converts covalently-closed circular, double stranded DNA, in a time and concentration dependent manner, to open circular (or nicked) and then to the linear form (double stranded break). The reaction does not occur unless a suitable reductant is present (Eichner, 1988). Based upon this knowledge, Tulluis (1986) has developed a method of making "footprints" of proteins bound to DNA. In his system, the hydroxyl radical, generated by the reduction of hydrogen peroxide by iron(II), is the reagent used to cut the DNA. Hydroxyl radicals break the backbone of DNA with almost no sequence-dependency. Since the DNA breaking by hydroxyl radical was found to be such an effective process, we decided to use this system to monitor, via DNA degradation, the level of free hydroxyl radicals. This
production directly correlates with the level of available iron ions in the immediate vicinity. The M-13 bacteriophage was chosen as the DNA source, enabling us to easily obtain both single and double stranded DNA from its two biological forms: the mature free virus, which is a single stranded circular DNA, and its replicative form inside the bacterial cell, which is a double stranded circular DNA.

Results

We have used M-13 bacteriophage DNA as the test system to monitor levels of redox-active available iron containing structures from parasitized erythrocytes, by estimating the amount of DNA degradation upon incubation at varying time intervals with lysates prepared from erythrocytes parasitized with mature forms or ring forms of *Plasmodium falciparum*. The degradation of DNA increases with time and is stage dependent, that is, extracts from mature forms are much more active as compared to extracts from erythrocytes infected with the ring form, and much more than extracts from free mature parasites. All experiments were conducted using both single stranded as well as double stranded DNA. Both single and double stranded DNA can be degraded in this redox-active system.

All the lysates, in each experiment, were prepared from the exact same culture, thus with the same parasitemia.

There was an enhancement in DNA degradation when ascorbate was added to the reaction mixture. There was no degradation
activity of lysates prepared from normal red blood cells, whether in the presence or absence of ascorbate. This suggests that iron within non-infected erythrocytes is not available for use in the redox-active system.

The DNA degradation by lysates of infected erythrocytes was inhibited by the chelator detapac. However, it was not affected by the iron chelator desferrioxamine, or by the enzyme catalase, suggesting that the iron ions participating in this degradation process are not free soluble iron ions, but possibly iron in the hemin structure. The results are similar for DNA degradation induced by hemin. As shown for extracts of parasitized erythrocytes, ascorbate enhances the DNA degradation which was catalized by hemin. In the hemin system, the chelator detapac protects the DNA from degradation while desferrioxamine and catalase do not, as in the experiments with lysates.

In order to further elucidate the role of iron-containing structures in the hydroxyl radical production, we measured reduction of ascorbic acid in the presence of lysates of parasitized erythrocytes as compared to hemin. The rate of ascorbate reduction was several times faster upon the addition of either hemin or infected erythrocyte lysate. Additionally, detapac inhibited ascorbate consumption in both systems while desferrioxamine had no effect.

One obvious possibility for DNA degradation could be the presence of an endogenous DNase in the parasite. In order to differentiate between such activity and the effect of the
hydroxyl radical, we abolished DNase activity by heat inactivation. DNA degradation was induced by parasitized erythrocytes even following the inhibition of DNase activity which was achieved by 65°C treatment for 10 min. This incubation completely abolished DNase activity in the free mature parasite as well as the activity of exogenous DNase added to normal red blood cells. We also found that when DNase was positively absent, as in the hemin system, there was no decrease in the efficiency of DNA degradation by heating. The results suggest that the observed DNA degradation is a result of iron ions participating in a Haber-Weiss reaction, producing free hydroxyl radicals that cause DNA breakage, and possibly involving a hemin or hemin-like system of the parasitized erythrocyte.

Discussion

Oxidative stress has already been incriminated as a deleterious factor in the development of the malaria parasite. However, the mechanism by which the parasite is affected by such a challenge needs further clarification. It has been previously shown that various chemical reductones (Chevion, 1982), which can undergo cyclic oxidation and reduction, can cause oxidative stress to infected red blood cells. Ascorbate, a naturally occurring redox-active compound, can act both as an anti and prooxidant. As the latter, it can induce the formation of active oxygen-derived species among which the hydroxyl free radical is the most deleterious species (Halliwell & Gutteridge, 1984). The
formation of hydroxyl is mediated by redox-active and available transition metals, iron and copper, via the metal-catalyzed Haber-Weiss reaction (Ausi et al., 1985).

During its development the parasite digests hemoglobin and sequesters large amount of heme in the form of malaria pigment (Stocker et al., 1985). This process could lead to the release of heme and probably other iron-containing products within the red blood cells (Habel, 1989). If these iron ions are available to the redox reaction, they should contribute to the increase of the free radical content, which in the presence of another redox-active agent (in a cell free system) will degrade DNA. In the present work it has been shown that while lysate from non-infected erythrocytes did not cause DNA degradation, lysate from infected ones did degrade both single and double stranded DNA. The amount of degradation was found to be dependent on the developmental stage of the parasite within its host; the more developed, the greater was the damage to the DNA. In another system it has been shown that free radicals are being formed and accumulated in the infected erythrocytes (Marva et al., 1989) without the addition of an exogenous redox-active agent. It was also found that the DNA degradation occurs whether ascorbate is added or not. This raises the possibility that an already existing endogenous reductone is participating in this reaction. Low levels of DNA degradation were also found to occur when lysate of the free parasite was incubated with DNA. Since lysates of red blood cells did not cause DNA degradation and
lysates of infected cells caused higher levels of DNA degradation than lysates of free parasite, it has been assumed that the degradation could partially be due to an endogenous DNase. However, heating to 65°C for 10 minutes completely abolished the activity of DNase that was added to normal red blood cells, as well as the DNA degradation activity observed with the free parasite lysate. Lysates of infected erythrocytes, upon heating to 65°C for 10 minutes, also lost part of the degradation activity. Thus, it seems that part of the activity is due to DNase but the remaining activity is due to the production of hydroxyl free radicals. This degradation activity was not affected by desferrioxamine, a very commonly used iron chelator, suggesting that the iron participating in the radical production is not in the form of free, soluble ions.

These results led to the hypothesis that there might be an iron structure where the transition metal can still undergo redox reactions. One such structure could be the hemin molecule. This idea was strengthened by further results, that demonstrated that hemin, in the presence of ascorbate, can degrade DNA, *in vitro*. These results are in agreement with others that have shown that hemin can cause denaturation of the free malarial parasite (Hebbel & Eaton, 1989). As with infected lysate, the DNA degradation in the hemin/ascorbate system was not inhibited by desferrioxamine or catalase but protected by detapac. In order to provide further evidence, ascorbate oxidation was measured in the presence of hemin or infected erythrocyte
lysate. Similar results were obtained. It is important to note that DNA degradation in the hemin/ascorbate system was not affected by heating to 65° for 10 minutes. The results are in accord with the assumption that the heating step did not affect the production process of free radicals or the subsequent DNA degradation.

It is suggested that oxidant stress leads to the destruction of the parasite in a multiple mode of action. The induction of the oxidant stress could be mediated by redox-active hemes that are being supplied in an increasing amount by the maturing parasite, which cause hemoglobin denaturation. We have also shown that measuring levels of DNA degradation could be a suitable way of estimating hydroxyl radical production and iron availability in plasmodial components.
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 \( \text{H}_2\text{O}_2 \). 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.
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 antioxidant 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 prooxidant 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 cooper 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 parasite within erythrocytes (viability was measured by hypoxanthine 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 against 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.

The effects of ascorbate on parasites in normal or G6PD-deficient erythrocytes was also compared. Pretreatment of G6PD-deficient erythrocytes or treatment of parasitized G6PD-deficient erythrocytes resulted in increased damage to *P. falciparum*.

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. The results demonstrate again the advantage of a G6PD-deficient individual over a G6PD normal one, in relation to malaria.

**Correlation Between Destruction of Malarial Parasites by Polymorphonuclear Leucocytes and Oxidative Stress**

The role of oxygen radicals in protection against malaria infections is in debate. However, there are many findings indicating the importance of leucocyte oxidative burst. This study evaluates the specific destructive role of ROS generated by PMNs.
An increase in the number of PMNs in human subjects infected with *P. vivax* or *P. falciparum* has been noted. Similar results were found in rodent models. In all cases PMNs counts returned to normal after recovery. The chemotactic activity of PMNs obtained during infection was not affected by the general phenomenon of immunosuppression during malaria attack. Moreover, it was found the PMNs from infected children are metabolically more active than normal PMNs.

It has been shown that simulated PMNs can affect *P. falciparum* development *in vitro*. However, various free radical scavengers and protective enzymes could not alter this deleterious effect. It was also found that PMNs from chronic granulomatous disease (CGD) patients, which are defective in their respiratory burst, can still inhibit parasite development. It should be also noted that PMNs can phagocytose and destroy various stages of malaria parasites. As PMNs are disintegrating after 5-7 hours of *in vitro* incubation, their degradation products may also kill the parasites (imitation of extracellular degranulation) and lead to uninterpretable results. Such experiments should be short in order to enable the discrimination of the specific role of ROS.

In view of these inconsistencies we were aiming to evaluate the idea that ROS induced by PMNs are involved in host defense against malaria.
Results

A. Establishment of the experimental system

The basic experimental system includes parasitized erythrocytes (PE) incubated with PMNs. Parasite development was evaluated by \(^{[3]}\text{H}\)-hypoxanthine (Hx) incorporation. The PMNs reduce Hx incorporation in a dose response manner. For each PMN/RBC ratio the effect is more pronounced in the second day.

PMNs undergo a progressive disintegration in culture within 32 hours. This may lead to release of intracellular proteolytic enzymes, which in turn, could affect parasite growth. In order to evaluate this destructive effect of disintegrated PMNs on Hx incorporation, we compared the effect of intact and viable PMNs with that of PMN extracts, both incubated with PE for 24 hours. The effect of the extracts was higher than that of the intact PMNs. This led us to examine the duration of the experiment. PMNs were incubated together with PE for various periods. After 1 hour there was a substantial effect. However, the radioactive counts were low. Also, the Hx incorporation was probably more influenced during the first hour by conditioning of the cells to the culture. This led us to choose a basic incubation period of 2-4 hours. Longer incubation periods were ruled out as the PMNs start to disintegrate after 5-7 hours. In six different experiments PMNs were incubated for 4 hours with erythrocytes parasitized with advanced forms of P. falciparum at PMN/RBC ratio of 1/30 and 1/90. The results show that there is a
variability in Hx incorporation in control and experimental cultures, and in the effect as expressed by the percent inhibition. However, for each single experiment the results were statistically significant.

B. The dependency of PMN effect on parasite developmental stage and type of host cell

(a) Pretreatment of non-infected erythrocytes. Normal or glucose-6-phosphate dehydrogenase (G6PD) deficient erythrocytes were prepared with PMNs (PMN/RBC ratio 1/30) in the presence of PMA, for 2 hours. The PMNs were separated from the erythrocytes on cellulose powder column. Subsequently, an inoculum of untreated parasitized erythrocytes was added to yield 1% parasitemia. The erythrocyte suspensions were divided into microplates and Hx incorporation was measured in two consecutive pulses of 15 and 21 hours. There was a marginal effect of PMNs within the first 15 hours on Hx incorporation by P. falciparum grown either in G6PD(+) or G6PD(-) erythrocytes. In contrast, during the following 21 hours (15-36 hours after inoculation), the effect of PMNs on parasites within both types of RBCs became marked, but more pronounced in the G6PD(-). Treatment of PE when compared to the pretreatment of non-infected RBCs with the same PMNs and PMN/RBC ratio yielded a much higher effect.

(b) Treatment of PE. Young stages are equally sensitive to PMNs if grown in either normal or in G6PD deficient erythrocytes. However, advanced stages are more sensitive to
PMNs when grown within G6PD deficient erythrocytes (at low PMN/RBC ratio). At PMN/RBC=1/10 there is often increased PMN aggregation which accelerates PMN disintegration and increases parasiticidal effects which are not associated with oxidant stress. Thus, at this ratio there is no difference in sensitivity.

C. Mechanistic effects

(a) Production of OH Radicals in Parasitized Erythrocytes. The conversion of added salicylate to dihydroxybenzoate (DHBA) was employed as a quantitative indicator for the produced fluxes of OH radicals. The level of DHBA in parasitized cells is higher than that in the non-infected cells (basal level). The incubation of non-infected RBC with PMNs increased DHBAs production by 3.4-4.3 fold. However, when PE were present, the increase was about 10 times, indicating an increased stimulation of PMNs by PE.

(b) "Contact Inhibition." Superoxide dismutase (SOD, 50 μg/ml) and/or catalase (90 μg/ml) could not alter the inhibitory effect induced by PMNs. This is in accord with Halliwell's suggestion (1990) that enzyme protection should not necessarily be expected when the effector cell and its target are in tight contact. We have designed experiments in which PE were kept separated from the PMNs by a 0.4 μ filter (1 mm distance). Identical chambers without the filters, where tight contact between PE and PMNs is possible, were used as control.
For comparison, the PMNs were also stimulated by PMA. PMA-stimulated PMNs were more active than non-stimulated PMNs. Both reduced Hx incorporation by PE, in a dose response manner. Separation between PE and PMN (whether PMA-stimulated or non-stimulated) completely reduced PMN effect, proving the close association needed for the expression of the damage.

(c) Discrimination Between Phagocytic Effect and the Results of the Respiratory Burst. PMNs were incubated with cytochalasin B for 5 minutes (initial cytochalasin concentration was 5 µg/ml), after which parasitized erythrocytes at the trophozoite stage (20% parasitemia) were added (PMN/RBC ratio was 1/90 in a final volume of 1 ml). The cultures were harvested after two hours pulse. At this cytochalasin concentration there is total inhibition of phagocytosis and 13% inhibition of Hx incorporation. The inhibition of incorporation by both PMNs and cytochalasin-B -- 36%, is close to the theoretical cumulative value of the combination of the separate effects of PMN and cytochalasin B. This result suggests that a substantial damaging effect is originating from the respiratory burst.

Discussion
The role of PMNs in eliminating malaria parasites has been well documented (Nnalue & Friedman, 1988). It has also been suggested that the PMNs activity is augmented by complement, antibodies and tumor necrosis factor (Kumaratilake et al., 1990). Several modes of action by which PMNs can eliminate the
parasite have been proposed: phagocytosis, ROS production, and the release of lysosomal components. Despite PMNs known potential to release ROS, the significance of this function in destruction of plasmodia has not yet been established. Moreover, various antioxidants could not reverse the inhibitory activity of neutrophils (Kharazmi et al, 1984). All other activities of PMNs are also augmented during respiratory burst, and PMNs defective in their oxidative metabolism could still inhibit the in vitro growth of *P. falciparum* (Kumaratilake et al., 1990). This inhibition is not evidence against an oxygen-dependent mechanism, it only shows that other mechanisms may also be involved.

This study confirms previous results which demonstrate a neutrophil-mediated protective response in malaria. The experimental system is only partially standardized: throughout the series of experiments we used the same strain of *P. falciparum*, which was grown in the same human plasma and batch of RPMI-1640. It was impossible to repeatedly use the erythrocytes or the PMNs of the same donor. Another factor that could interfere with reproducibility was that the erythrocytes could not always be used on the same day following their donation, and that the developmental stage of the parasite could not exactly be matched to previous experiments (i.e., the trophozoites could be mixed with early schizonts in one experiment, while in another one, there was only trophozoites). The effect of PMNs on parasite development varied from person to
person. Even the activity of PMNs donated from the same person on different days was not identical. However, in all cases, PMNs caused a profound significant reduction in the incorporation of Hx into *P. falciparum* grown in culture.

We were aiming at the evaluation of the discrete role of oxidant stress in inhibiting parasite development. The effect of PMNs in *P. falciparum* was already seen within 1 hour. However, due to low Hx counts during the first hour in culture, it was preferable to extend the experiments to 2-4 hours. It is possible that the parasites overcome some of the initial interference, however, most of the effect is not reversible during this period. Longer periods of incubation could lead to artifacts due to cytotoxicity associated with PMNs degradation products, or reflect proteolytic activity of the PMNs. Varani *et al.* (1989) found that target endothelial cells are killed by PMNs during the first 4 hours, mainly by oxygen products. If the time course for exposure of the target cells is extended to 18 hours, progressive injury is mediated by PMN proteases. It is possible that in the system of Varani *et al.*, as well as in ours, and as suggested by Weiss (1989), the ROS immediately damage the parasite, but also modify the parasitized erythrocyte to render it more sensitive to a subsequent effect of PMNs lysosomal products. It is unlikely that the PMNs reduced the viability of the parasites by competing with them for nutrients because of the short duration of the experiment, and due to the small amount of PMNs needed to inflict the damage: in some
experiments, 2 hours incubation of PMNs with parasitized erythrocytes at a ratio of 1/30 was sufficient to reduce Hx incorporation by 40%.

While phagocytosis was prevented by cytochalasin-B, PMNs still interfered with parasite development. Cytochalasin would not prevent the respiratory burst accompanied with extracellular release of ROS. Another indication for the involvement of ROS in our system is the increased effect of the PMNs on parasites developing in G6PD-deficient erythrocytes, since G6PD-deficient erythrocytes are more sensitive to oxidant stress (Nagel & Roth, 1989).

The PMNs influenced parasite development by virtue of a damage which had been exerted prior to the invasion (i.e., pretreatment of non-infected erythrocytes) or by virtue of changes induced by treatment of the parasitized erythrocytes. In both cases G6PD-deficient erythrocytes were associated with increased interference with parasite development. Pretreatment of the erythrocytes could change the fate of the parasites by both affecting the invasion and the preceding development, while treatment could damage the intracellular development. Thus, components of the immune system could play a selective role in evolution, providing an advantage to this otherwise deleterious type of erythrocyte. It was suggested that the oxidative stress is responsible for retention of the genetic features of both erythrocytes and effector cells -- by selecting various traits of erythrocytes associated with increased sensitivity to oxidant
stress (Allison, 1954), and by shaping the ROS-producing cytotoxic cells of the immune system (Schirmer et al., 1987 during evolution. Our results, which demonstrate the increased effect of PMNs in P. falciparum developing in G6PD-deficient erythrocytes, support the two parts of this hypothesis.

Catalase and SOD could not prevent the effect of PMNs. It has been suggested that this does not necessarily exclude ROS involvement (Halliwell, 1990). We found that a tight contact is needed between the PMNs and the PE, in order to inflict or initiate the injury. Protective enzymes may operate less efficiently in such a microenvironment. Another possibility is that following the contact between the effector and the target cells, ·OH radicals are produced within the parasitized erythrocyte (especially when the parasite is well developed and digestion of hemoglobin probably releases redox-active iron-containing structures (Har-El et al., in press)). It is unlikely that the protective enzymes can penetrate the parasitized erythrocyte and prevent internal radical production.

An additional substantiation for radical involvement is the generation of the highly reactive hydroxyl radicals. There is a general agreement that this radical is among the most reactive and deleterious ROS.

It has been speculated whether PMNs are capable of forming hydroxyl radicals. However, an ESR study shows ·OH production through Haber-Weiss or Fenton type reactions, requiring trace
iron either inside or outside the cell (Samuni et al., 1988). Indications for the progressive increase in redox-active iron during the growth of *P. falciparum* have been recently found in our laboratory (Har-El et al., in press). Thus, the iron may catalyze the production of the *·OH* radical in the plasmodial system. The increased level of hydroxylation of salicylate in a system containing PMNs activated by parasitized erythrocytes, suggests that *·OH* radicals produced by the Fenton reaction are involved in plasmodial killing. However, there is a variety of other cytocidal molecules which could participate in eliminating malaria parasites and have not yet been investigated in this context. Likewise, neutrophils also contain the enzyme myeloperoxidase, some of which is released during activation. Myeloperoxidase catalyzes an H₂O₂-dependent oxidation to halide ions to produce OCI⁻. The hypochlorite ion exists in equilibrium with its protonated form (HOCl). Both are powerful oxidizing agents that can attack a wide range of biomolecules.

It should be noted that phagocytic cells, including neutrophils, are capable of producing nitric oxide (Rockett et al., 1991). The nitric oxide and related molecules, the nitric oxide radical (NO⁻) and its oxidized forms nitrite and nitrate (termed reactive nitrogen intermediates) have antiplasmodial activity. We present circumstantial evidences that there is a correlation between the in vitro destruction of *P. falciparum* by the PMNs and the oxidative stress. Other
factors (which may interact with the ROS) could also be involved.

Reduction and Oxidative radical reactions
of Selected Antimalarial Drugs

The design of new antimalarial compounds or derivatives of already existing drugs that were efficient in the past, depends on understanding the mechanism of their action. However, the mode of action of many important common drugs (AMD) is not defined. It has been proposed that some antimalarial drugs may undergo oxidation/reduction cycling (Vennerstron & Eaton, 1988). In this respect we have investigated three AMDs that have been in use for a long time, but whose mechanism of action is debatable -- quinine, chloroquine and primaquine.

Quinine, isolated from the bark of the Cinchona tree, was for decades an effective chemotherapeutic drug against malaria, but its use declined between the 1940s and the 1960s, due to the introduction of new, effective synthetic antimalarial drugs, such as quinacrine and chloroquine. Chloroquine, less toxic than quinine, has been used clinically to treat malaria, rheumatoid arthritis and collagen vascular disease (Rollo, 1980). However, since the early 1960s, there has been a worldwide resurgence of malaria infections, due in part to the appearance of chloroquine-resistant strains, and as a consequence, quinine has
once again been employed. Biochemically, the mechanism of antimalarial action by chloroquine is not understood. Some contend that chloroquine, by its accumulation in the parasite food vacuoles, binds the ferriprotoporphyrin, and it is the resultant product that is toxic to the parasite (Fitch, 1983), whereas others suggest that the drug acts directly on enzymes present in the parasite food vacuole to inhibit their action (Yayon et al., 1984). Chloroquine concentrations which are deadly to \textit{P. falciparum} in vitro and in vivo, cannot affect the pH in the food vacuole. thus, the assumption that a pH change is responsible for "starvation" of the parasite is not likely, and the target of chloroquine should be searched for elsewhere (Ginsburg et al., 1989). Meshnick (1990) suggests that chloroquine's antimalarial effect is due to its ability to intercalate into the parasite DNA. Slater and Cerami (1992) suggest that chloroquine is active by inhibiting a specific plasmodial metabolism -- the heme polymerase activity, which is responsible for the polymerization of toxic heme, to a non-toxic hemozoin.

Primaquine can induce favism-like effects in G6PD-deficient erythrocytes and oxidant stress in G6PD(+) parasitized erythrocytes, probably due to its influence on the hexose monophosphate shunt, leading to increased production of NADPH (Deslauriers et al., 1987). It was suggested that primaquine can also form a charge transfer complex with NADPH or NADH, leading to the oxidation of the reduced pyridine nucleotides and
generation of active oxygen species, which in turn may affect
the sensitive erythrocytes (Augusto et al., 1986). Fletcher
et al. (1988) suggest that primaquine by itself has no
significant oxidant effect on normal and G6PD-deficient
erythrocytes. In contrast, its metabolites, 5-hydroxy and
5,6-dihydroxy derivatives, caused an increase in methemoglobin
and a decrease in GSH levels (the relation to malaria is
reviewed by Golenser et al., 1983). Bisby (1988) uses pulse
radiolysis to produce one electron-reduced product of primaquine
and to examine various reactions of this radical species. He
suggests that primaquine radicals play a less significant role
in the aerobic oxidation of NAD(P)H and oxygen radicals
production, and consequently these antiparasitic effects of
primaquine are less than was previously supposed.

Under physiological conditions, a significant fraction of
quinine is found in an uncharged form, which allows it to
penetrate into lipid bilayers. The quinine produces noticeable
changes in the structure of lipid side chains of a model
bilayer. Thus, it was speculated that quinine may exert its
antiparasitic effect by altering substrate binding for
phospholipases in the food vacuole of the parasite (Zidovetzki
et al., 1983). Other possible mechanisms for quinine activity
are unknown.

In view of the structure similarity of chloroquine, quinine
and primaquine, and the fact that primaquine can induce oxidant
stress, we examined whether single electron redox reactions of
the three drugs contribute to the understanding of their antimalarial properties.

**Pulse Radiolysis Studies**

Radical production, by definition, always involves single electron reactions. Amino-quinoline compounds can theoretically be reduced by hydrated electrons (e_{aq}^-), and oxidized by hydroxyl ('OH) or azide ('N_3) radicals. In the earlier studies, primaquine was reduced, and its reduction of the paraquat cation, NAD^+ and oxygen determined (Bisby, 1988). Amodiaquine, in contrast, was oxidized to the phenoxy radical, and its repair by Trolox-O and ascorbate was evaluated (Bisby, 1990). Unfortunately, the results from both studies are compromised by the fact that kinetic spectroscopy was limited to the visible region between 400 to 700 nm.

We decided to study both reductive and oxidative radical reactions of the AMDs chloroquine (CLO), primaquine (PRIM) and quinine -- first to observe the transient spectra of the individual radicals and second, to determine their reactivities vis-a-vis likely in vivo substrates. As shown for the transient spectra, after e_{aq}^- attack and OH, respectively 'N_3 attack, our own spectral cut-off was only influenced by the far-uv absorption of the parent substances (Ssrar et al., 1982). Clearly distinct transient spectra are observed for each of the AMDs after the individual reductive and oxidative univalent reactions.
We found that CLO is most effectively reduced by \( \text{e}_\text{aq}^- \) (it is also the only one of the three AMDs which was reduced by the formate radical, \( \text{CO}_2^- \)), whereas PRIM is the best scavenger of the oxidizing radicals, \('\text{OH}' \) and \('\text{N}_3' \). Unfortunately, investigations of quinoline model compounds have never been performed (and only 8-amino and 6-methoxy derivatives are commercially available), and it is thus difficult to assign the sites of attack to specific structural moieties, as has been done for the phenoxy radical of AMQ (Bisby, 1990). However, based on spectral parameters of the parent compounds (CRC Handbook of Chemistry and Physics, 1986), at least the absorption minima at 330-340 nm, observed for CLO after attack of all three radicals, can be assigned to the 4-amino group rather than the 7-chloro atom. Due to less apparent bleaching, such an assignment is more difficult with PRIM (we propose predominant attack at the 8-amino group) and almost impossible for quinine.

This generation and absorption of the AMD radicals was, however, of secondary interest, since such species cannot be detected in vivo. In fact, we were far more inquisitive about the reactivity of these radicals with biological substrates. Two models of action can be envisaged.

1. Transfer of reducing equivalents from a reduced radical to oxidized metal compounds, thus making these, catalysts for Fenton chemistry.

2. An oxidized radical conversely oxidizes "bireductants", thus depleting the cell of reducing equivalents, respectively
generating oxidized derivatives symptomatic of oxidative stress.

We determined the reactivities of the reduced AMD radicals towards model ferric and cupric complexes, possible precursors for Fenton-chemistry catalysts, calculated from the pseudo-first order decay of the AMD radicals at various concentrations of the metal complexes. The rate constants for the oxidized AMD radicals with the bioreductants ascorbate, glutathione and NADPH were determined at non-competitive concentrations of these substrates (at higher concentrations they would merely scavenge \(\cdot\)OH radicals themselves and thus diminish the absorption signal due to the [AMD(OH)]\(^\cdot\) radical.

Mode (1) has previously been shown to operate for the neurotoxin MPP\(^+\) (Bors et al., 1990), with the even more severe restriction for AMD\(^\cdot\) radicals, that due to the extremely negative redox potential it seems unlikely that such an univalent reduction occurs in vivo. Mode (2) was not observed with MPP\(^+\), yet it is very much apparent with all the three AMDs.

Aside from thermodynamic restrictions in case of the reactions of the reduced AMD radicals, kinetic considerations may still limit the significance of the rate constants in view of the instability of these radicals. Since all [AMD(OH)]\(^\cdot\) radicals decay by second-order reaction (except for a first-
order decay of \([\text{CLO(OH)}]^\cdot\) at pH 6.0), this pathway would become less significant at low steady-state concentrations of the radicals -- thus favoring reactions with non-radical substrates; provided that these are present in sufficient amounts. Calculations of such reaction probabilities contains data for pH 6.0 and 8.5, since the pH in the food vacuole of malaria parasite, where some AMDs accumulate, may change (Krogstad & Schlesinger, 1987; Ginsburg et al., 1989).

It is obvious from these calculations that \([\text{PRIM(OH)}]^\cdot\) is most likely to oxidize each of the bioreductants at both pH=4, whereas \([\text{QUIN(OH)}]^\cdot\) may do this only with ascorbate and NADPH (and GSH at pH 8.5).

Correlation of Pulse Radiolysis with in vivo Data

What has to be kept in mind if we try to correlate these data with in vivo evidence on the reactions of these drugs is the dichotomy between drug action against parasite infection and drug toxicity against host organs or tissues. Evidently, CLO is least capable of inducing redox reactions, in line with the fact that the toxicity of this drug is low (Peterson et al., 1990). Primaquine, as well as its hydroxylated metabolites, are the most toxic of the three compounds investigated (Price & Fletcher, 1986). This suggests that the apparent readiness of PRIM to directly oxidize bioreductants may contribute to both drug action and drug toxicity -- and not only formation of
reactive oxygen species (Thornalley, 1983; Augusto et al., 1986) of protein adducts via the quinone imine or depletion of GSH (Link et al., 1986; Maggs et al., 1988).

It has repeatedly been proposed for CLO that the sites of attack are membrane structures of both host and parasite (Ngaha et al., 1985). While membrane damage could be accounted for by the generation of oxygen radicals, this aspect has only been verified for PRIM (Augusto et al., 1986), and is considered an unlikely prospect to explain CLO drug action/toxicity. First of all, the extreme negative reduction potential of -1.83V for the free base of CLO (Amas et al., 1987), exceeding the values of all known bioreductants (Linkous et al., 1988), makes it unlikely that such a radical is ever produced in vivo, an argument previously raised for the neurotoxin MPP⁺. Furthermore, a prerequisite for O₂⁻ production is the presence of oxygen, in essence a contradiction to the anaerobic conditions necessary to produce the reduced CLO radical in the first place. It is obvious from these arguments that we do consider reduction of oxidized metal complexes less likely to contribute significantly to the overall mechanism of AMD.

In contrast, the parasite-induced degradation of hemoglobin to hemin/ferriprotoporphyrin (Chou & Fitch, 1980) or to aggregates, the so-called malaria pigment (Yayon et al., 1984), and the subsequent interaction of hemin with CLO (Sugioka et al., 1989) do cause oxidative stress, and may thus explain the drug action of CLO (Fitch, 1989). Interestingly, the
requirement of parasites for iron, evident from the antimalarial effect of desferrioxamine, is satisfied by extracellular iron rather than the host heme iron (Pollack, 1989). Our findings that the combination of Cu\textsuperscript{2+} and ascorbate optimally inhibits the development of \textit{P. falciparum} parasites in G6PD-deficient erythrocytes, also suggests the involvement of reduced metal complexes -- but not necessarily in conjunction with AMD -- in oxidative stress conditions detrimental to the parasites (Eaton \textit{et al.}, 1976).

Related to the antimalarial reactions of Cu\textsuperscript{2+}/ascorbate are the observations on the reactions of another Cu\textsuperscript{2+} chelator, diethyldithiocarbamate (DDC). Originally, a synergistic effect with PRIM was explained by the pro-oxidant effect of the drug (\textit{vide supra}) and inhibition of the antioxidative enzyme SOD by DDC ((Meshnick \textit{et al.}, 1986). This effect may be potentiated if, as reported for the murine strain \textit{P. berghei}, the parasite contains no SOD of its own, and adopts the host enzyme (Fairfield \textit{et al.}, 1986). Recently, the antimalarial function of DDC was reinterpreted as forming a toxic Cu\textsuperscript{2+}-DDC complex, even with intracellular copper sources (Meshnick \textit{et al.}, 1990). However, as the lysis of erythrocyte membranes by such a complex was not narrowed down to any specific mechanism, this hypothesis remains doubtful as it neglects an earlier report on oxidative effects on DDC in erythrocytes -- GSH depletion, methemoglobin formation -- aside from inactivation of SOD (Kelner \textit{et al.}, 1986).
Despite the low toxicity of CLO as compared to PRIM (Petersen et al., 1990), the resistance of malaria strains against this AMD being an entirely different problem (Newbold, 1990). CLO may not be such an innocuous drug after all. The two defense systems, (i) oxidative stress conditions leading to reactive oxygen species (Vennerstrom et al., 1988), or lipid peroxidation products (Clark et al., 1987), both toxic to plasmodia, and (ii) the immune system (Nielsen & Theander, 1989), can be compromised by CLO. The second case has been mentioned earlier (Foutagne et al., 1989) and may involve receptor interaction rather than inhibition of the NADPH oxidase, either directly or indirectly, via protein kinase C inhibition. The first case, however, may be even more serious as CLO actually inhibits the antioxidative enzymes themselves (Nair et al., 1989). Due to an adverse reaction, this was considered a bonus in disguise, as by impairing this host defense system CLO would render erythrocytes more susceptible to oxidative stress and consequently more resistant towards parasite infection.

Of the three AMDs investigated, QUIN has been used the longest, but it is still the least known. With a reduction potential of -1.63 to -2V for the free base (Ames et al., 1985), it is equally unlikely to form a reduced radical \textit{in vivo} (Linkous et al., 1988). According to our data, the oxidation capabilities are arranged in the order \([\text{CLO(OH)}]^+<[\text{QUIN(OH)}]^+<[\text{PRIM(OH)}]^+\), in line with the absence of any reports on oxidation reactions of QUIN \textit{in vivo}. It
furthermore shows little photo toxicity in erythrocytes (Ealing & Sibley, 1987), and its influence on rat polymorphonuclear leukocyte function is far minor or even opposite to CLO, AMQ or MFQ — e.g., no effect on PMN migration, minor inhibition of \( \text{O}_2^\cdot \) production, and inhibition rather than stimulation of lysosomal enzyme release. It does, however, in its uncharged form, penetrate lipid membranes and alter the lipid side chains in model bilayers, leading to the suggestion that during its drug action it may affect substrate binding to phospholipases (Zidovetzky et al., 1989).

**Conclusions**

We were able to show the transient spectra after univalent reduction or oxidation of the three most common antimalarial drugs chloroquine, primaquine and quinine (only for primaquine an earlier pulse radiolysis study has been reported). Rate constants for the generation of these radicals by hydrated electrons, hydroxyl or azide radicals, are all in the diffusion-controlled range. Despite fast decay rates, these radicals are kinetically capable to transfer their electron equivalents to oxidized metal complexes (for the reduced AMD radicals), respectively to bioreductants such as ascorbate, glutathione and NADPH (in case of the oxidized AMD radicals). The latter reaction, in particular for primaquine, is a likely explanation for both its drug action and toxicity towards host tissues. For the less toxic chloroquine and quinine, alternative mechanisms
are more reasonable, which at least in the case of chloroquine involve interaction with hemin as degradation product of host hemoglobin or the host defense system.

Collaborative Efforts

Prof. J.L. Ngu visited the Hebrew University in 1990. The aims of his visit were to discuss further possibilities of submitting a common grant proposal(s) which would enable us to continue our collaboration and to evaluate the immediate tasks of the Cameroonian trainee, Mr. Appolinaire Tsafack, in Israel.

Following the visit of Prof. Ngu, new preproposals were written and submitted to AID/CDR. The research goals of these preproposals, as formulated by participants in the meetings (Prof. J.L. Ngu, Prof. M. Chevion, Prof. J. Golenser and Dr. S. Frankenburg) are related to cell-mediated immunity and cerebral malaria. since then, appropriate full proposals were prepared. It was agreed that the AID/CDR grant C7-163, would serve as seed money for continuous cooperative research. Thus, it is extremely important for the continuation of the scientific collaboration, that such proposals will be funded. This will provide an excellent chance for exchange of knowledge and information between the Israeli and Cameroonian labs, and will enable us to explicit our common experience, investigate new aspects related
to malaria and oxidant stress, cell-mediated immunity and antimalarial design.

The work in Cameroon was devoted to the relationships between oxidant stress and malaria. It is reflected in the thesis of Mr. A. Tsafack (see also in the previous section, and items relating to the effects of paraquat, ascorbate and PMN on malaria; in which Mr. Tsafack was involved). The prevalence of G6PD-deficiency and hemoglobinopathies in Yaounda was evaluated. It was found that these genetic traits are more prevalent in Yaounda than in people from traditionally non-endemic areas. The results are summarized in an attached report and in the thesis of Mr. L.R. Bifusina (presented in partial fulfillment of the requirements for post-graduate diploma in biochemistry).

Collaborative Study on the Protective Mechanisms Against Malaria Associated with G6PD-Deficiency

Summary of Research Activities in Cameroon

G6PD-deficiency is widespread, but particularly so in areas where malaria is endemic. It is thought that malaria has exerted a selective pressure, not only on this enzyme deficiency which is a genetic disorder, but also on other genetic hemoglobinopathies, such as HbS and thalassemia. Individual with G6PD-deficient red cells seems to have an advantages as regards malaria infections.
Our collaborative project with the Kuvin Centre in Israel seeks to understand the mechanisms of resistance to malaria associated with G6PD-deficiency. Malaria parasites would grow in both normal and G6PD-deficient erythrocytes, but in the presence of oxidant stress, parasite development in G6PD-deficient cells is inhibited. The mechanism are not clearly understood. A comparison of the effects of different forms of oxidant stress (food toxicants, drugs, immunological mechanisms) on the growth of malaria parasites in normal and deficient erythrocytes would throw some light on this.

We have therefore continued here in Cameroon to screen for the prevalence of G6PD, thalassemia and Hbs, and have kept on with trying to establish in vitro cultures of \textit{P. falciparum}. A graduate student, Mr. Tsafack Appolonaire, spent one year in Israel, from November 1989 to December 1990.

**Screening of G6PD Deficiency**

A screening of G6PD deficiency has been carried out in 184 students and staff of our institution. The kit of Biomerieux in France was used.

According to this method normal values are from 3.3-5.5 u/g Hemoglobin. Following the classification by Bowman and Murray (1990) we have classified the individuals as follows:

- **Increased enzyme activity** >5.5 u/g Hb
- **Normal enzyme activity** 3.3-5.5 u/g Hb
- **Moderately decreased enzyme activity** 2.3-3.3 u/g Hb
Intermediate enzyme activity 1.0-2.3 u/g Hb
Severe enzyme defect 0-1.0 u/g Hb

The results obtained are as follows:

<table>
<thead>
<tr>
<th>Number of Individuals (%)</th>
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<tbody>
<tr>
<td>1. Increased enzyme activity 8 4.4</td>
</tr>
<tr>
<td>2. Normal enzyme activity 71 38.6</td>
</tr>
<tr>
<td>3. Moderately decreased enzyme activity 42 22.8</td>
</tr>
<tr>
<td>4. Intermediate enzyme activity 42 22.8</td>
</tr>
<tr>
<td>5. Severe enzyme defect 21 11.4</td>
</tr>
<tr>
<td>Total 184 100.0</td>
</tr>
</tbody>
</table>

**Hemoglobinopathies**

Screening for thalassemia was carried out using DE52 chromatography. The percentage of HbA2 was measured in 101 individuals. This technique specifies that normal percent A2 values in adults are 1.5 to 3.5%. Our values were mostly far beyond 1.5%. In our 101 samples there was none positive for thalassemia.

Screening HbS was carried out by electrophoresis on cellulose acetate paper. The results show that 43 out of 184
individuals were HbAS. This gives a percentage of 23.37%. No homozygous individuals were found.

**Plasmodium falciparum Cultures**

We have continued trying to establish cultures, though with lots of difficulties. We have had a major problem getting sera to use for cultures. We are hoping that this problem would be solved by our Israeli counterparts.

**Report on Training Period of Mr. A. Tsafack in Jerusalem**

The aim of the training of Mr. A, Tsafack was to study techniques which will be applied in Yaounda, after his return to Cameroon. another aspect was to enable him to actively participate in research activities of the malaria unit of the Hebrew University-Hadassah Medical School.

Techniques of malaria culture, diagnosis, electrophoresis, examination of the effects of antimalarial drugs *in vitro* and *in vivo* (in animal models), enzyme detection (i.e., superoxide dismutase), transport of metabolites, cell-mediated immune responses, and computer graphics and statistical analysis, were studied.

Mr. A. Tsafack has proved himself as a highly intelligent student, hard working and rather independent scientist. He participated in the current research in the lab and will be an
author of a paper focusing on the role of polymorphonuclear cells in destruction of malaria parasites by oxidant stress.

Mr. Tsafack was always most welcome in our laboratories; his willingness to help whenever needed and his pleasant nature, rendered him a highly liked individual by the members of our group. He will apply for a scholarship that will enable him to conduct additional research in Israel. We fully support this idea and hope that if accomplished it would add further strengthening to the cooperation between the scientists from Cameroon and Israel.

Meanwhile Mr. Tsafack has finished his thesis on "Free Radicals and Malaria: A Study of the Effects of Oxidative Stress on the Growth of P. falciparum in vitro, and of P. berghei in vivo." This work has been completed in Yaounda and was presented in partial fulfillment of the requirements towards "Doctoral de 3e Cycle" in Biochemistry.
List of Papers Related to the Project

Oxidant stress and malaria: host-parasite interrelationships in normal and ab normal erythrocytes. A review.

Deleterious synergistic effects of ascorbate and copper on the development of P. falciparum: an in vitro study in normal and in G6PD deficient erythrocytes.

The effect of ascaridole on the in vitro development of P. falciparum.

Bors, W., Golenser, J., Chevion, M., Saran, M. 1990.
Reductive and oxidative radical reactions of selected antimalarial drugs.
International conference on "Free Radicals and Biology", Pasadena, USA.

Effect of sinefungin on macromolecular biosynthesis and cell cycle of P. falciparum.

typing of Southern African isolates of P. falciparum using monoclonal antibodies.

The effects of free radicals induced by paraquat and copper on the in vitro development of P. falciparum.

Induction of oxidant stress by iron available in advanced stages of P. falciparum.

Survival of plasmodia under oxidant stress. A review.
Parasitol. Today 7:142-146.

Free radicals and malaria. A review.
P. falciparum: evidence for a DNA methylation pattern. 

The effects of ascorbate-induced free radicals on P. falciparum 

Golenser, J., Kamyl, M., Tsafack, A., Marva, E., Cohen, A., 
Correlation between destruction of malarial parasites by 
polymorphonuclear leucocytes and oxidative stress. 

Golenser, J., Chevion, M. 
Implications of oxidant stress and malaria. A review. 

Har-El, R., Marva, E., Chevion, M., Golenser, J. 
Malaria derived redox active iron containing structures play an important role in inducing oxidant stress. Hemin is a possible candidate. 