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IS HEMIN RESPONSIBLE FOR THE SUSCEPTIBILITY OF PLASMODIA TO OXIDANT STRESS?

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Based on the unusually high and stage-dependant susceptibility of Plasmodia to oxidant stress it has been proposed that during parasite development, increasing levels of redox-active forms of iron are gradually released. The purpose of this study was to examine this proposal by using an assay monitoring the levels of available forms of iron for redox reactions. Ascorbate-driven and iron-mediated degradation of adventitious DNA served as the basis for this functional assay.

Incubation of DNA with lysate from infected RBC caused massive degradation, which was dose, time- and parasite-stage dependent. In contrast, lysate from non-infected RBC did not induce DNA degradation. Likewise, lysate only from infected RBC enhanced the aerobic oxidation of ascorbate. These effects on both reactions, DNA degradation and ascorbate oxidation, could be reconstructed with hemin, instead of lysate. Also, chelators exerted similar effects on both reactions.

The results suggest that increased levels of redox-active forms of iron are liberated during parasite development. We propose that hemin or hemin-like structures are the appropriate candidates which could catalyze oxidative stress and deregulate the delicate redox balance of the host-parasite system.

KEY WORDS: Malaria, Free radicals, Hemin, DNA degradation

Abbreviations: DETAPAC: Diethylenetriamine pentaacetic acid, DS DNA: Double stranded DNA, EDTA: Ethylenediamine tetraacetic acid, RF: Replicative form, SS DNA: Single stranded DNA, TBE: 0.1M TRIS (pH = 8.3), 10 mM Boric acid, 2.5 mM EDTA

INTRODUCTION

Malaria parasites are very sensitive to the toxic effects of reactive oxygen species (ROS), such as superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH\cdot$) which are found within parasitized erythrocytes [1,2]. These active oxygen species may be generated by various chemical reductones [3,4] which can undergo cyclic oxidation and reduction, such as dialuric acid/alloxan [5], isouramil [6], divicine [7] or ascorbate [8]. Additionally, all of the above have been shown to cause oxidative damage to infected red blood cells. It is widely accepted that hydroxyl radicals can cause cellular damage [9] and that its formation in various biological systems is mediated by redox-active and available iron and copper [10].

Previously it has been shown [8] that the addition of ascorbate to synchronized cultures, at later stages of parasite development, suppressed parasite development.

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This could be mediated by newly released intracellular iron (from a yet undefined source) that could enhance an oxidant stress.

In the present study, evidence is provided that increased levels of an endogenous redox-active agent(s), are released upon the parasites' maturation.

Within the past decade it has been established that cells are exposed to a continuous flux of oxyradicals, produced by aerobic cells. As a result, oxygenated and hydroxylated derivatives of DNA bases are produced at high levels [11]. Thus, radical damage to DNA is an ongoing event of life that cells must cope with [12,13]. One possibility is that hydroxyl radical reactions with DNA are preceded by association of metal ions to the DNA molecule, forming a complex, followed by the production of the hydroxyl radical, at a particular site. This theory has been named "site specific DNA damage" [10,14–18]. Although most oxyradicals do not interact with DNA, the hydroxyl radical does [11]. 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). It is also the most electrophilic radical. The hydroxyl radical is so reactive that it can only diffuse 5–10 molecular diameters before it reacts [13] so if it is not produced near the DNA it probably will not react with it.

DNA is not efficiently cleaved by a non-redox-active metal induced mechanism [19]. However, DNA degradation by radicals formed in metal catalyzed autooxidation of ascorbate (or other reducing agent) is well known [20]. This metal catalyzed process could nick circular, double stranded DNA, to the relaxed circular form and then to the linear form in a time and concentration dependent manner. This type of reaction does not occur unless a suitable reductant is present [21]. Based upon this knowledge, Tullius et al. [22] have developed a method for "footprinting" 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 dependence. Since DNA breaking by hydroxyl radical was found to be such an efficient process, it seemed suitable as a system to monitor the level of free hydroxyl radicals. This latter production should correlate with the level of available iron ions in the immediate vicinity. M-13 bacteriophage was chosen as the source of DNA. Both single and double stranded DNA could be easily obtained 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 [23].

MATERIALS AND METHODS

Blood

Blood was collected from normal healthy individuals and stored at 4°C. Experiments were initiated within 48 h following collection of the blood. The blood was centrifuged and buffy coat was removed.

Parasites

Plasmodium falciparum (strain FCR-3) was cultured according to the method of Trager and Jensen [24], in RPMI-1640 medium (Gibco) supplemented with 20 mM glucose, 100 μg/ml gentamycin, 25 mM HEPES, 2 g/liter $NaHCO_3$, with some modifications as earlier described [6]. Cultures were synchronized by sorbitol treatment [25]. Ring forms were collected and 24 h later the mature forms were collected from the same culture.

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Strains and Maintenance

JM103 is an *Escherichia coli* K-12 derivative. For short term storage (2–4 weeks at 4°C) bacteria were plated on M-9 minimal plates [12] supplemented with 0.2% glucose as a carbon source. M-13 bacteriophage served as a source of DNA and was maintained either as a free virus or as its replicative form inside the bacteria.

DNA Preparation

Single and double stranded DNA were purified from the two biological forms of M-13. The circular single stranded DNA of the phage was isolated from the mature free phage according to a modification of Shreier and Cortese [26]. The supercoiled double stranded DNA was isolated from infected bacteria containing the M-13 replicative form according to Felsenstein protocol [27]. After isolation of DNA it was further purified on a pZ523 column, and residual contaminating RNA was removed by RNAse treatment as described by the manufacturer (5 Prime→3 Prime, Inc.).

Lysate Preparation

Non-infected normal erythrocytes or erythrocytes parasitized with either ring or mature forms at parasitemia of 15–50%, were washed twice with isotonic phosphate buffer saline (PBS) (pH = 7.2), supplemented with 20 mM glucose, at a cell concentration of 10^9/ml. Cells were lysed by freezing and thawing. A portion of erythrocytes parasitized with mature forms was treated with 0.05% saponin in growth medium for 5 min at room temperature, in order to release free mature parasites. The free parasites were also lysed by freezing and thawing.

Hemin Preparation

Hemin (Type III) (Sigma) was prepared by dissolving in 50 mM sodium hydroxide.

DNA Degradation Reactions

In general, ~2 μg of single stranded or ~7 μg of double stranded DNA were incubated with lysates of infected erythrocytes (10% hematocrit, containing ring or mature forms), or of non-infected erythrocytes or of free mature parasites, for 60 min (or as indicated in the Legends), at 37°C, in a 30 μl total volume, in PBS, supplemented with 20 mM glucose. Following incubation, the samples were transferred to an ice water bath. Five μl of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol and 30% glycerol) were then added and samples were electrophoretically run on agarose gel.

Agarose Gel Electrophoresis

25 μl DNA mixtures were run on 1% agarose gel in Tris-borate/EDTA Buffer, pH = 8.3 (TBE). Electrophoresis was performed at 20 V for 20 h. Gels were stained at the end of the run with Ethidium Bromide (0.5 μg/ml) in TBE buffer, for 30 min. Photographs were taken with a Polaroid Land Camera using Polaroid 667 film.

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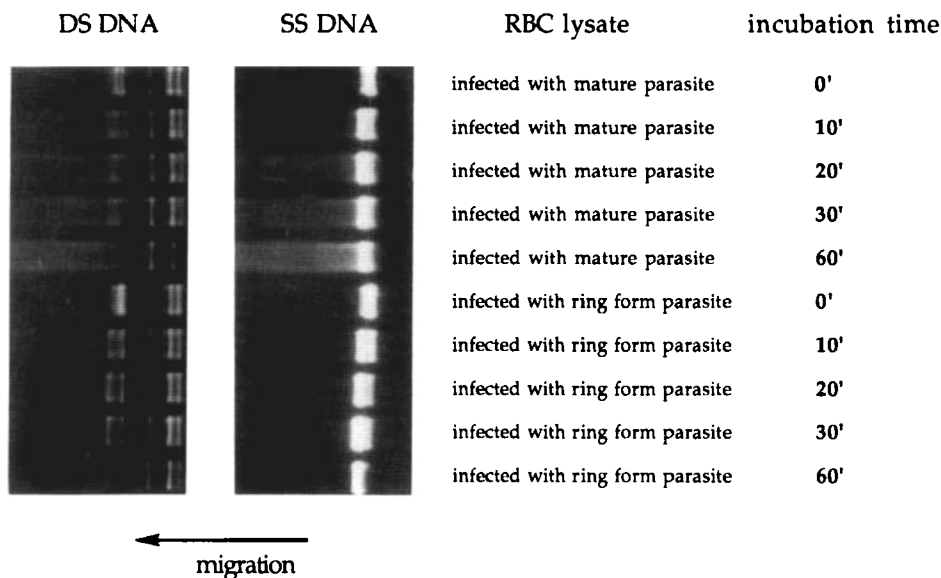

FIGURE 1 Degradation of single and double stranded DNA in the presence of parasitized erythrocytes. ~ 2 μg of single-stranded DNA or ~ 7μg double-stranded DNA of the bacteriophage M13 were incubated with lysates (25-fold diluted) of erythrocytes infected with either mature (lanes 1-5) or ring form (lanes 6-10) of the parasite (at 15% parasitemia) in PBS (pH = 7.2), for different time intervals, as indicated, at 37°C. At the end of incubation, samples were transferred to an ice-water bath, then run on an agarose gel as described in Materials and Methods.

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Ascorbate Oxidation Reactions

Ferriphenanthroline assay for the determination of ascorbate oxidation was conducted according to Chevion et al. [28]. Stock solution of ferriphenanthroline was diluted 1:10 in imidazole buffer (0.1M, pH = 8.0). 1 ml aliquots were transferred to optical cells (one at a time), 50 μl of ascorbate oxidation reaction mixture were added and optical density was recorded at 515 nm wavelength, for 10 min.

Each experiment was repeated at least three times and representative experiments are presented.

RESULTS

We have monitored the degradation of bacteriophage DNA in order to estimate the levels of available and redox-active iron within parasitized erythrocytes. Figure 1 shows degradation of both single and double stranded DNA upon its incubation at varying time intervals with lysates prepared from erytrocytes parasitized with either mature or ring forms of *Plasmodium falciparum*. Degradation of both single and double stranded DNA increased with time and was stage dependent. Lysates of erythrocytes infected with mature forms were much more active. All experiments were conducted using both single and double stranded DNA and similar results were obtained for both.

Figure 2 shows the lysate dose response on DNA degradation efficiency. Lysates

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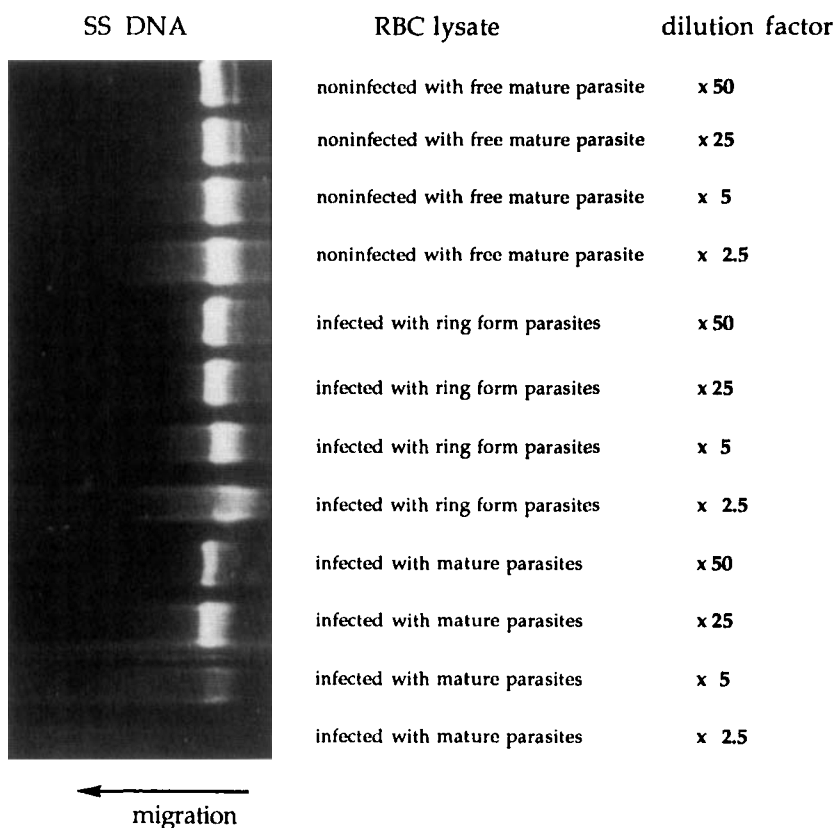

FIGURE 2 A dose dependent effect of lysate on DNA degradation. ~ 2 μg of single stranded DNA were incubated for 60 min at 37°C in PBS (pH = 7.2) with lysates from either non-infected RBCs together with free parasites (lanes 1-4), erythrocytes infected with ring (lane 5-8), or with mature forms (lanes 9-12) (at 15% parasitemia), at different lysate concentrations (50, 25, 5 and 2.5-fold diluted, respectively). Degradation reactions were terminated and samples were run on agarose gel, as described in Materials and Methods.

prepared from erythrocytes infected with mature forms, had higher degradative activity as compared to lysates from erythrocytes infected with ring forms, and much greater than the degradative activity measured with lysates of non-infected erythrocytes together with free mature parasites which had been released from infected cells. In each experiment, all the lysates, originated from the same culture (with the same parasitemia).

Figure 3 shows an enhancement in DNA degradation following addition of ascorbate to the reaction mixture. Additionally, it demonstrates the lack of degradation activity by lysates prepared from non-infected red blood cells, whether in the presence or absence of ascorbate. This suggests that iron within non-infected erythrocytes is not available for redox reactions.

Figure 4 compares the DNA degradation by lysates of parasitized erythrocytes and hemin. The degradation in the hemin-containing system occurred only in the presence of ascorbate. In contrast to the results obtained with the lysates from the parasitized

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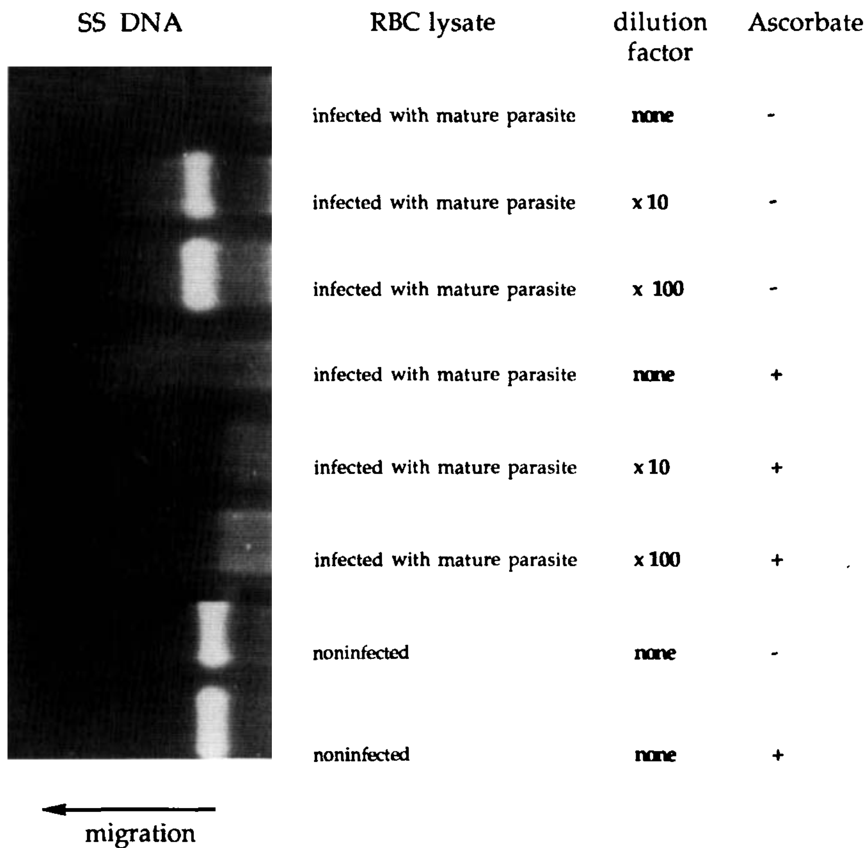

FIGURE 3 Ascorbate-driven and parasitized erythrocytes lysate-mediated DNA degradation. ~2 μg of single stranded DNA were incubated for 60 min at 37°C with non-diluted (lanes 1,4); 10-fold dilution (lanes 2,5); 100-fold dilution (lanes 3,6) lysates of erythrocytes infected with mature forms (at 50% parasitemia), or with non-diluted non-infected red blood cells (lanes 7,8) in the absence (lanes 1-3,7) or presence (lanes 4-6,8) of ascorbate (2.5 mM). Degradation reactions were terminated and samples were run on agarose gels, as described in Materials and Methods.

erythrocytes where ascorbate was not obligatory, but increased the effect of lysates from erythrocytes infected with mature forms, as shown earlier. Figure 4 also shows that the DNA degradation which is mediated by either hemin or lysates is equally inhibited by the chelator DETAPAC. However, it is only slightly affected in either system by the iron chelator desferrioxamine or catalase (the result with catalase was inconsistent, and in some of the experiments, catalase protected against the degradation).

In order to further investigate the similarity between the lysate and the hemin systems we measured oxidation of ascorbate in their presence. Figure 5 demonstrates that the rate of aerobic oxidation of ascorbate is enhanced upon the addition of either hemin or infected erythrocyte lysates. DETAPAC inhibited ascorbate oxidation in both systems (80-90%), while desferrioxamine had a much lower effect on both.

One obvious possibility for DNA degradation could be the presence of an endogenous DNase in the parasite. We ruled out the possibility that the effects are

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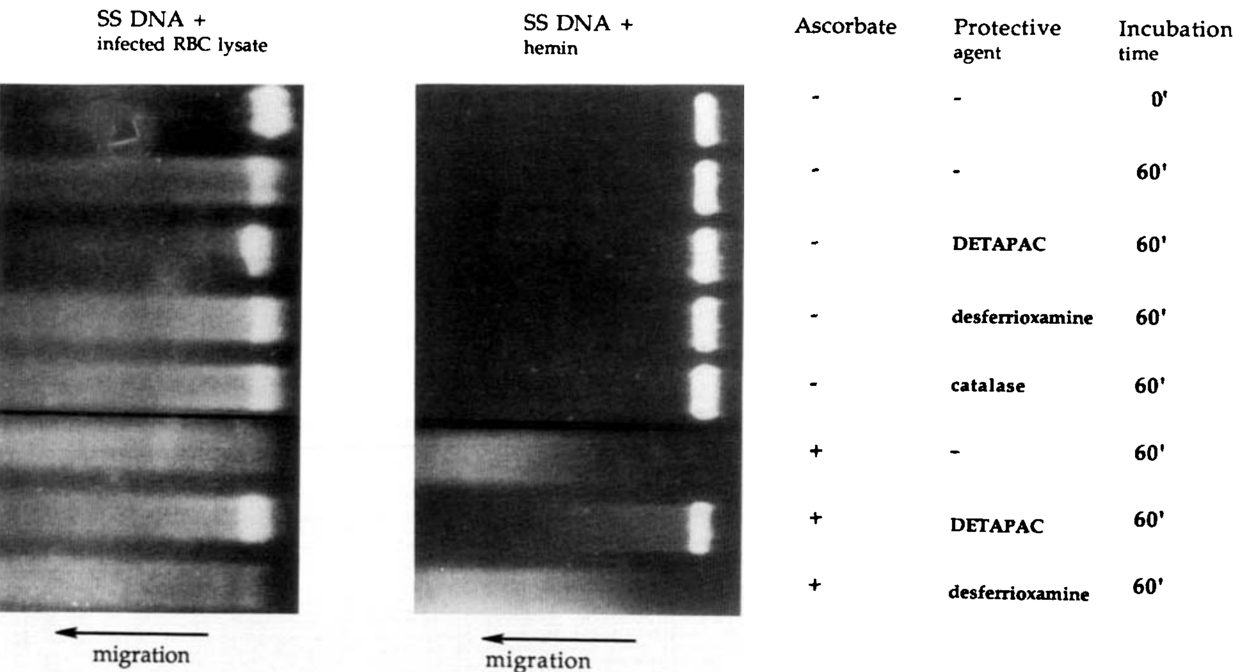

FIGURE 4 Ascorbate-driven parasitized erythrocytes lysate- or hemin-mediated DNA degradation. ~2 μg of single stranded DNA were incubated for 0 (lane 1) or 60 min (lanes 2–8) at 37°C with either, lysates of erythrocytes infected with mature forms (2.5 fold diluted, at 50% parasitemia), or with hemin (100 μM). Ascorbate (2.5 mM) was added to the reaction mixtures, in lanes 6–8. DETAPAC (1 mM) (lanes 3,7); Catalase (100 μg/ml, 40,000 units/mg protein) (lane 5) or Desferrioxamine (1 mM) (lanes 4,8) were preincubated with the lysates or hemin for 15 min, at 37°C, prior to the addition of DNA and ascorbate. Degradation reactions were terminated and samples were run on agarose gel as described in Materials and Methods.

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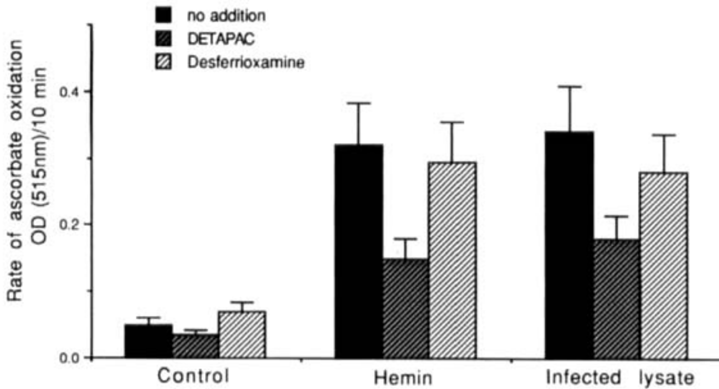

FIGURE 5 Rate of aerobic oxidation of ascorbate in the presence of either hemin or lysate of infected erythrocytes. Hemin (100 μM) or non-diluted lysate from erythrocytes infected with mature forms (at 50% parasitemia) were preincubated with or without DETAPAC (100 μM) or Desferrioxamine (100 μM) for 5 min, at 37°C. Ascorbate (0.5 mM) was added and aliquots (50 μl) were immediately transferred to optical cells containing 1 ml of ferriphenanthroline reaction mixture. Samples were then incubated for 10 min, at 37°C and changes in optical density were monitored at 515 nm as described in the Materials and Methods.

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mainly due to the presence of DNase from a parasitic source. The DNase activity could be abolished by heat treatment in both free mature parasites, as well as in adventitious DNase I, added to non-infected red blood cells. DNase I was completely inactivated by incubation at 65°C for 10 min [29]. Figure 6 shows that in the presence of lysates, increased degradation takes place, even following the inhibition of endogenous DNase activity. Additionally, it was demonstrated that in the absence of DNase, as in the hemin system, there is no decrease in the efficiency of DNA degradation by heating (Figure 6). Malaria parasites contain various proteases and hemoglobinases. Thus, heme might be released during cell lysis as a result of protease activity. As control, extracts of parasitized erythrocytes were also prepared in the presence of protease inhibitors (dithiothreitol and phenylmethyl-sulfonyl fluoride) and heated for 10 min at 65°C. Degradation was only slightly affected by the inhibition of proteases (data not shown). The overall conclusion is that most of the observed DNA degradation is a result of iron ions participating in redox reactions producing hydroxyl radicals that cause DNA breakage, and possibly involve a hemin or hemin-like species which originate from the parasitized erythrocyte.

DISCUSSION

Oxidative stress has already been incriminated as a deleterious factor in the development of the malaria parasite [reviewed in 8,30–34]. 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 [3,4] which can undergo cyclic oxidation and reduction, can cause oxidative stress in infected red blood cells. Ascorbate, a naturally occurring redox-active compound can act both as an antioxidant as well as a pro-oxidant. The latter can induce the formation of active oxygen-derived species including hydroxyl radicals, which are the most deleterious ones

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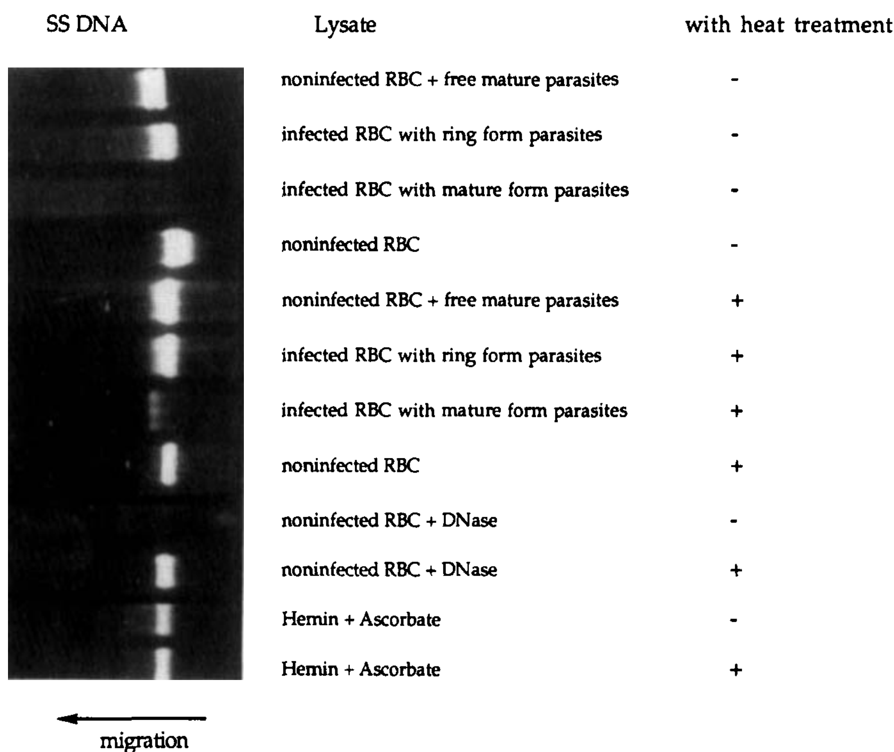

FIGURE 6 DNA degradation activity following heat inactivation. ~2 μg of single stranded DNA were incubated for 60 min, at 37°C, with lysates (2.5-fold diluted) of either, non-infected RBCs together with free mature parasites (lanes 1,5), or erythrocytes parasitized with ring forms (lanes 2,6), or mature forms (lanes 3,7), or non-infected red blood cells (4,8,9,10), or with hemin (100 μM) and ascorbate (2.5 mM) (lanes 11,12); with 10 min preincubation of the lysates at 65°C (lanes 5-8,10,12), and in the presence of adventitious DNase I (100 μg) which was added prior to this preincubation (lanes 9,10).

(9). The formation of hydroxyl radicals is mediated by redox-active and available transition metals, iron and copper, via the Haber-Weiss reaction [10,35].

During its development, the parasite digests hemoglobin [36,37] and sequesters large amounts of heme in the form of malaria pigment [38]. The limited fidelity of this process could lead to the release of low levels of heme and probably other iron-containing products within the infected red blood cells [39]. In the presence of a reducing agent such iron ions are available for redox reactions, leading to an increase of a cellular flux of free radicals, as shown here, by the in vitro degradation of adventitious DNA. Lysates from non-infected erythrocytes did not support DNA degradation, but lysates from infected erythrocytes enhanced the degradation activity of both single and double stranded DNA. The efficiency of degradation was dependent on the developmental stage of the parasite; the more developed, the greater was the damage to the added DNA. It was previously shown that the hydroxyl radicals are formed in the infected erythrocytes even without the addition of an exogenous reducing equivalent [2]. Analogously, in the present work it was found that DNA degradation occurs whether ascorbate is added or not. These findings suggest that an endogenous reductone can drive this reaction. Low levels of DNA degradation were

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also found to occur when lysate of the free parasite was incubated with DNA. It was assumed that part of this degradation could be due to a parasite endogenous DNase. Incubating lysates of free parasites at 65°C for 10 min completely abolished their DNA degradation activity as well as that of an exogenous DNase added to non-infected red blood cells. In contrast, lysates of infected erythrocytes lost only a portion of their degradation activity, after incubation at 65°C for 10 min. Thus, it is assumed that only a small fraction of this activity was due to endogenous DNase, while the major part of the degradative activity was due to the metal ion-mediated redox reactions. Addition of protease inhibitors to parasitized erythrocytes before the lysis of the cells did not change the degradative activity of the extract, ruling out the possibility that plasmodial proteases are solely responsible for releasing heme from hemoglobin.

Desferrioxamine, a commonly used iron chelator [40], slightly protected against ascorbate-driven and infected-erythrocyte lysate mediated DNA degradation, suggesting that the iron participating in the free radical production was not in a "free" Fe(III) state. DETAPAC, another commonly used chelator for iron, better protected against DNA degradation. These results suggest that an iron-containing, redox-active structure had been liberated. One such structure could be hemin or a hemin-like molecule.

Direct spectrophotometric measurements of solutions containing hemin and either of the two chelators show that while desferrioxamine did not affect the spectral characteristics of hemin, DETAPAC caused significant changes in the Soret Band and in the 530–650 nm region (data not shown). These changes could indicate axial interaction between the ferric porphyrin iron and the carboxylic groups of DETAPAC. This proposal is further substantiated by the similarity in the ascorbate-driven DNA degradation activity mediated by lysate of infected erythrocytes as compared to that mediated by hemin. Finally, the aerobic oxidation of ascorbate and the effects of desferrioxamine and DETAPAC on its rate, in the presence of either hemin or infected erythrocyte lysate, were found to be similar.

These results are in agreement with the findings that hemin could cause damage to erythrocytes [39] and denaturation of malaria parasites [41]. The presence of iron-containing structures in parasitized erythrocytes was also associated with the activity of antimalarial drugs: a reaction between artemisinin and hemin induced the oxidation of protein thiols in erythrocyte membranes [42]. This may explain the selective toxicity of the drug against malarial parasites [42]. Slater and Cerami [43] 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. Thus, chloroquine may increase oxidant stress by promoting the accumulation of redox-available iron in hemoglobin derivatives. In this work it was found that even a hemin concentration as low as 10 μM was sufficient to induce degradation in the hemin/ascorbate/DNA system (data not shown). It has been found that addition of chloroquine during parasite maturation induces the release of heme in concentrations which are also in the range of 10 μM (H. Ginsburg, Dept. of Biological Chemistry, Institute of Life Sciences, Hebrew University of Jerusalem, Jerusalem, Israel, personal communication). This recent finding supports the idea that heme is involved in the susceptibility of plasmodia to oxidant stress.

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 degrades hemoglobin. Monitoring the levels of adventitious DNA

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degradation could serve as a suitable and sensitive assay for estimating hydroxyl radical production and iron availability in plasmodial components.

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