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INDUCTION OF OXIDANT STRESS BY IRON AVAILABLE IN ADVANCED FORMS OF *PLASMODIUM FALCIPARUM*

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Oxidative stress has been incriminated as a deleterious factor in the development of malaria parasites. Various chemical reductones which can undergo cyclic oxidation and reduction, such as ascorbate have been shown to cause oxidative stress to red blood cells. This, naturally-occurring and redox-active compound, can induce the formation of active oxygen derived species, such as superoxide radicals ($\cdot O_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH\cdot$). The formation of the hydroxyl radical, the ultimate deleterious species, is mediated by the redox-active and available transition metals iron and copper in the Haber-Weiss reaction.

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

KEY WORDS: *Plasmodium falciparum*, malaria, iron, oxidant stress, free radicals.

INTRODUCTION

During the course of its development the plasmodia profoundly alters the structure and function of their host erythrocyte.[1] It can be assumed that these alterations are inflicted on the erythrocyte in order to support the development and survival of the parasite – mainly by allowing the influx of vital nutrients and the secretion of toxic catabolites.[2] Along the development of the parasite, the alterations of its host become more significant. In addition, an oxidant stress which is induced by the parasite becomes more pronounced.[3]

The production of increased amounts of reactive oxygen species (ROS) by the

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parasite[4,5] depletes the erythrocyte of its defense mechanisms: superoxide dismutase (SOD), catalase, glutathione peroxidase, NADPH, NADH, GSH, and glutathione reductase.[3] As oxidative stress is exerted by the growing parasite, in cells which are highly sensitive to such a challenge, the enhanced alterations could result in a retarded development of the parasite. Thus, the coexistence of both parasite and erythrocyte is a matter of a delicate balance. Modulations in this state such as a host cell which is more sensitive to oxidant stress, additional stress originating from external sources (food constituents or drugs) or induced by the immune system — may interfere with the successful development of the parasite.[1,3,6]

During the development of the parasite, hemoglobin is progressively digested and a concurrent release of high levels of iron-containing breakdown products takes place within the red blood cell. This increase in redox-active iron may affect the development of the parasite and also some host components.[1]

MATERIALS AND METHODS

Parasite Development

Incorporation of radioactive hypoxanthine (Hx) served as a parameter for parasite development: Hx (18.5 kBq/well, New England Nuclear), was added in 25 μl of medium to the culture. The cells were collected by filtration on glass microfibre filters and radioactivity was counted. The incorporation of Hx in non-infected erythrocytes did not exceed 1–3% of the lowest level of uptake by parasitized red blood cells. Parasitemia was estimated by using Giemsa stained blood smears.

DNA Preparation

M-13 bacteriophage served as a source of DNA and was maintained as a replicative form inside *Escherichia coli* (JM103, K-12 derivative) or as a free virus. The bacteria were plated on M-9 minimal plates.[7] Single-stranded DNA was purified from the mature virus. The circular single-strand DNA of the phage was isolated from the mature free phage according to Shreier and Cortese.[8]

Nicking Reaction

Non-infected normal erythrocytes or erythrocytes parasitized with ring forms (25% parasitemia) or mature forms (15% parasitemia), collected from the same culture 24 h later were washed twice in isotonic PBS (pH = 7.2). 100 μl aliquots of the 10% suspensions were lysed by freezing (−70°C) and thawing (room temperature). 1–2 μl DNA (1 mg/ml) were mixed with 20 μl of the lysates and incubated at 37°C for 1, 30 or 60 minute intervals. The reaction was terminated by the addition of 10 mM desferrioxamine and the samples were transferred to 4°C. 3 μl of loading buffer (0.25% Bromo Phenol Blue, 0.25% xylene cyanol, 30% glycerol) were then added and the samples were run on agarose gels.

Agarose Gel Electrophoresis

10 μl DNA reaction mixtures were run on 1% agarose gel in Tris-borate/EDTA

(TBE) buffer (pH = 8.3). Electrophoresis was performed at 100 V for 6 hours. Gels were stained at the end of the run with 0.5 μg/ml Ethidium Bromide in TBE buffer, for 30 minutes. Photographs were taken with a Polaroid Land Camera using Polaroid 667 film.

RESULTS

Hypoxanthine Incorporation

Synchronous cultures of red blood cells parasitized with *Plasmodium falciparum* were exposed to ascorbate, and hypoxanthine (Hx) incorporation was monitored for the first and the second day following this treatment (Table I). In the first day, ascorbate (1–9 mM) slightly enhanced Hx incorporation (6–14% enhancement). In contrast, in the second day, ascorbate caused a marked dose dependent suppression of Hx incorporation (9–91% suppression).

Infected Erythrocytes as a Source of Redox-Active Iron

Hemolysates of normal erythrocytes or erythrocytes infected with ring forms or mature forms of *P. falciparum* were incubated with viral DNA for 1, 30 or 60 minute intervals. The samples were examined by agarose gel electrophoresis (Figure 1). No degradation of viral DNA was observed after incubation of lysates from non-infected erythrocytes (NRBC) with the DNA. Hemolysates of synchronous cultures of erythrocytes infected with ring forms of *P. falciparum* (RING) slightly supported DNA degradation. In contrast, hemolysates of red blood cells infected with advanced forms (TR = trophozoite) markedly promoted the breakdown of DNA.

DISCUSSION

Various chemical reductones which can undergo cyclic oxidation and reduction, such

TABLE I
The effect of ascorbate on hypoxanthine incorporation into erythrocytes parasitized with *P. falciparum*

Time of harvest*	Ascorbate (mM)	Hx Incorporation (cpm)**	Change (%)
19 h	0	24,904	–
	1	26,544	+ 6
	3	27,736	+11
	9	28,361	+14
43 h	0	20,958	–
	1	19,151	– 9
	3	16,704	–21
	9	2,016	–91

Ascorbate was added to cultures of *P. falciparum* (1% parasitemia, 5% hematocrit), and remained in the medium.
*[^{3}H] hypoxanthine (Hx) was added 12 h prior to the harvest of the culture.
**Average of triplicates.

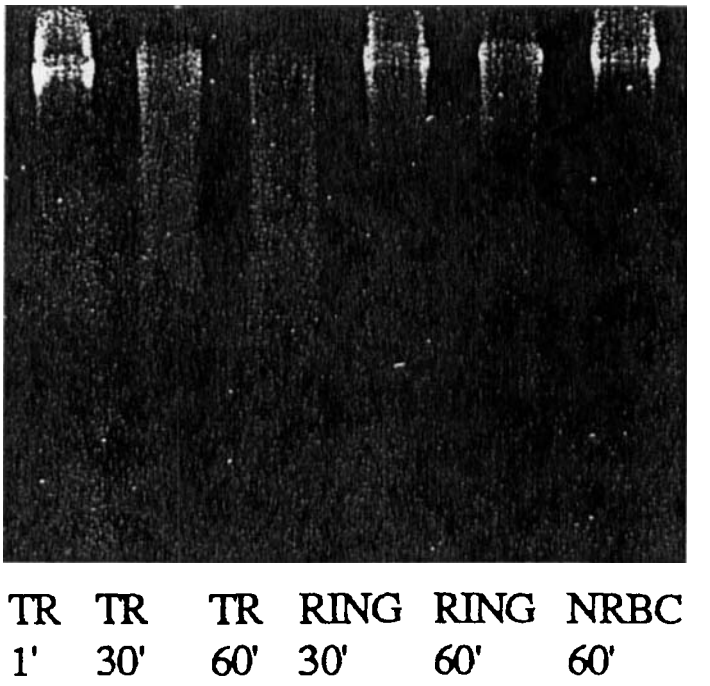

FIGURE 1 Degradation of DNA, induced by redox-active iron present in parasitized erythrocytes.

as dialuric acid/alloxan,[9] isouramil,[10] divicine[11] or buytl hydroperoxide[12] have been shown to cause oxidative stress in red blood cells. Likewise, ascorbate, a naturally-occurring redox-active compound, can induce the formation of active oxygen-derived species. It was suggested that the hydroxyl radical could be the ultimate deleterious species[13] and that its formation is mediated by the redox-active and available transition metals, iron and copper.[14,15]

We found that ascorbic acid caused a destructive effect on the *in vitro* development of *P. falciparum*. The fact that ascorbate proved highly detrimental to the plasmodia in the absence of an external source of transition metal and only when added to the mature forms of the parasite (developing in the second day of culture) suggests that there is a progressive increase in redox-active iron during the intracellular development of *P. falciparum*. Hemoglobin is progressively digested and a concurrent release of high levels of iron-containing breakdown products takes place within the red blood cell. It has been suggested that traces of redox-active and available iron can catalyze the transformation of a superoxide radical anion ($\cdot O_2^-$) to the highly reactive hydroxyl radical ($OH\cdot$), via the metal catalyzed Haber-Weiss reaction.[13,14]

Experiments were carried out to examine whether the infected erythrocytes can serve as a source of redox-active iron for the ascorbate-driven metal-mediated degradation of DNA. We found that erythrocytes containing advanced stages of the parasites were much more active than erythrocytes containing young ring forms. In contrast, no degradation was observed after incubation of lysates of non-infected erythrocytes with the DNA. These results may also indicate the increase in redox-

active iron during the growth of *P. falciparum*. Several studies support the existence of parasite proteases capable of degrading the erythrocytic hemoglobin.[16] The degradation of host cell hemoglobin provides a major source of amino acids for the growth of the parasite,[17] but may also supply increasing amounts of iron. We conclude that there is a progressive increase in redox-active iron during the development of *P. falciparum* in the erythrocytes.

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