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GLUTATHIONE STABILITY AND OXIDATIVE STRESS IN P. FALCIPARUM INFECTION IN VITRO: RESPONSES OF NORMAL AND G6PD DEFICIENT CELLS

Eugene F. Roth, Jr.[#], Carmen Raventos-Suarez*, Margaret Perkins[+] and Ronald L. Nagel*

[#]Mount Sinai School of Medicine, Department of Medicine, Polly Annenberg Levee Hematology Center, One Gustave L. Levy Place, New York City 10029 USA, *Albert Einstein College of Medicine, Division of Experimental Hematology, Department of Medicine, Bronx, New York 10461 USA, [+]Rockefeller University, 1230 York Avenue, New York City 10021 USA

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SUMMARY: Red cell oxidative stress in P. falciparum infection in vitro was investigated in relation to the G6PD-Malaria hypothesis. Glutathione stability was enhanced in infected red cells; glucose consumption and pentose pathway activity were not different in normal and G6PD deficient cells, although parasite growth was impaired in G6PD deficiency. Evidence for a response to oxidative stress was not found. Infected red cells have glutamate dehydrogenase activity which was not found in uninfected cells. This enzyme provides a separate pathway for the generation of NADPH independent from the pentose shunt. The data suggest that a significant oxidative stress is not present in falciparum malaria and that another mechanism may be operative in G6PD deficiency.

Red cell glucose 6 phosphate dehydrogenase (G6PD) deficiency has been postulated for many years on epidemiologic grounds to offer protection against lethal infection with P. falciparum malaria (1). Using in vitro culture techniques for P. falciparum, it was possible to demonstrate in an unequivocal manner that red cells from G6PD deficient Sardinian subjects impaired the growth of malaria when compared to appropriate controls (2). Nevertheless, the biochemical mechanism whereby this inhibition is produced remains obscure.

Oxidative stress is usually invoked as the relevent mechanism for growth inhibition of P. falciparum in G6PD deficient cells, but direct evidence in human erythrocytes infected with this species of malaria is scarce (3).

These studies were designed to detect red cell responses to oxidative stress in P. falciparum infection in vitro, and to contrast the findings with those found in classical acetylphenylhydrazine oxidative stress. An unambiguous oxidative stress of this kind is well known to stimulate the pentose shunt

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pathway and compromise reduced glutathione (GSH) stability (4). Since GSH stability depends in part on NADPH generation, the possible presence of an alternate source for this compound was of great interest.

MATERIALS AND METHODS: Plasmodium falciparum (FCR-3 Strain) was obtained through the kindness of Dr. James Jensen and maintained in culture by the method of Trager and Jensen (5). Blood samples were obtained in standard CPD solution from normal controls and 5 G6PD deficient donors. The donors consisted of 4 black persons (3 male, 1 female) with G6PD acitivity 5-15% of the normal value, and 1 male of Persian-Jewish ancestry with no detectable red cell G6PD activity by kinetic spectrophotometric assay using Sigma Corp. Kit No. 345 UV (Sigma Co. St. Mo. USA). Infected cells were concentrated by gelatin sedimentation (6). Control cells were subjected to all the same procedures.

Glutathione stability at 37°C was evaluated by placing cell suspensions in either complete growth medium, isotonic phosphate buffered saline, pH 7.4 or RPMI medium without glucose at a final concentration of acetylphenylhydrazine of 2.5 mg/ml. Packed red cell volumes varied from 10-15%. At the times indicated, 0.2 ml aliquots were withdrawn for estimation of reduced glutathione (GSH) by the method of Beutler (7).

Glucose consumption and pentose shunt activity were measured as described previously (8). Briefly, total glucose remaining in the supernatant was determined by means of Sigma Kit No. 15 UV. Pentose shunt activity was monitored by measuring the evolution of $^{14}CO_2$ from ^{14}C-1-labelled glucose (New England Nuclear Co., Boston, Mass. USA) specific activity 45 Ci/mol (9). Red cell glucose consumption was expressed as mM glucose consumed/Liter-RBC's/hr. Glucose metabolized via the pentose shunt was expressed as percent of the total glucose consumed and as micromoles glucose/Liter-RBC's/hr.

The Electrophoretic Demonstration of Glutamate Dehydrogenase: Glutamate dehydrogenase is an oxidoreductase and a deaminating enzyme which utilizes NADP and converts glutamate to alpha ketoglutarate and ammonia with reduction of NADP to NADPH. This enzyme is not present in uninfected human erythrocytes. The enzyme was demonstrated by starch gel electrophoresis by a minor modification of the method of Fine and Costello (10) and Momen (11).

Red Cell Alpha Ketoglutarate Measurements: Activity of glutamate dehydrogenase in red cells in the direction in which NADPH is generated should result in the production of alpha ketoglutarate. The content of red cell alpha ketoglutarate was determined by the enzymatic method of Bergmeyer (12).

RESULTS: 1. Glutathione content and GSH stability: In 5 separate experiments using normal blood with parasitemias of 24-81%, there was no significant difference in GSH content between infected and control red cells from the same donor (Table I).

In 4 separate experiments using acetylphenylhydrazine, infected normal red cells displayed a greater GSH stability than control cells. At 45 minutes incubation with acetylphenylhydrazine, there remained 1.8 times more GSH in infected red cells (Fig. 1A). When glucose was omitted from the suspending medium, GSH was slightly more unstable in the infected cells (Fig. 1B).

TABLE I

REDUCED GLUTATHIONE (GSH) CONTENT OF NORMAL HUMAN RED CELLS INFECTED WITH P. FALCIPARUM MALARIA

	GSH μmole/gm Hb.		
	Control	P. falciparum infected	% Parasitemia
1.	3.9	3.5	81
2.	5.6	6.1	24
3.	4.6	4.2	75
4.	4.7	4.2	25
Mean ± S.D.	4.7 ±0.7	4.5 ±1.1	51 ±31

Because infected red cells contain a high content of methemoglobin, another control was run with normal cells in which 100% methemoglobin had been induced with 1% $NANO_2$. It was found that the presence of large amounts of methemoglobin enhanced the disappearance of GSH (data not shown). Thus, methemoglobin should produce GSH instability--exactly the opposite of what was found. Taken together, the results suggest that the metabolic activity of infected red cells and their parasites were required to produce the enhanced GSH stability which was observed.

2. Glucose consumption and Pentose shunt activity: Infection of red cells in vitro increased total glucose consumption markedly (Table II). Total glucose utilization was increased up to 9-fold depending on the parasitemia.

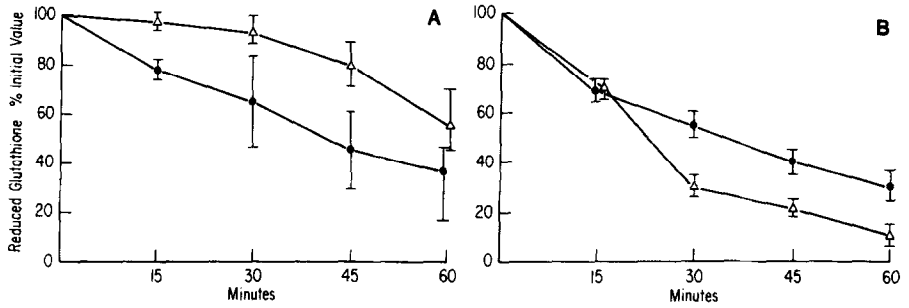


Figure 1A: Reduced glutathione stability of normal human erythrocytes infected with P. falciparum (parasitemia 25-81%); control and infected red cells were adjusted to a packed cell volume of 10-15% in complete culture medium (5) and placed in shaking erlenmeyer flasks at 37°C with 2.5 mg/ml acetylphenylhydrazine. At indicated times, 0.2 ml aliquots were removed for determination of glutathione (7). Results show mean ±S.D. for three separate experiments. Circles: uninfected control RBC's; triangles: P. falciparum infected RBC's.

Figure 1B: Reduced glutathione stability of normal human erythrocytes infected with P. falciparum. Conditions are identical to those for Fig. 1A except that glucose has been omitted from the suspending medium. Results show mean ±S.D. for three separate experiments.

TABLE II

HEXOSE MONOPHOSPHATE SHUNT ACTIVITY OF NORMAL AND G6PD DEFICIENT RED CELLS INFECTED WITH FALCIPARUM MALARIA

	Parasitemia Mean ±S.D.	Total Glucose Consumed mM/L-RBC's/hour Mean ± S.D.	% via Shunt	Micromoles of Glucose via Shunt per L-RBC's/hour Mean ± S.D.
Normal control (4)	-	1.5±0.5	5.7±1.8	85.5± 27
P. falciparum infected normal (4)	9.1±1.0	13.4±3.4*	3.0±1.1[#]	402 ±147.4[@]
G6PD Def. control (5)	-	1.3±0.6	5.1±3.2	64.6± 30.3
P. falciparum infected G6PD Def. (5)	4.9±1.4	8.0±3.5**	2.0±0.6[#]	160.5± 70.0[b]
Normal extrapolated to 100% parasitemia	100	147.2±37.3	3.0±1.1	4416 ±1619
G6PD Deficient extrapolated to 100% parasitemia	100	163.7±71.4[#]	2.0±0.6	3274 ±1438[#]

[#] N.S.
* p<.001
** p<.010
[@] p<.025
[b] p<.05

TABLE III

THE EFFECT OF ACETYLPHENYLHYDRAZINE ON GLUCOSE CONSUMPTION AND PENTOSE SHUNT ACTIVITY IN NORMAL HUMAN RED CELLS

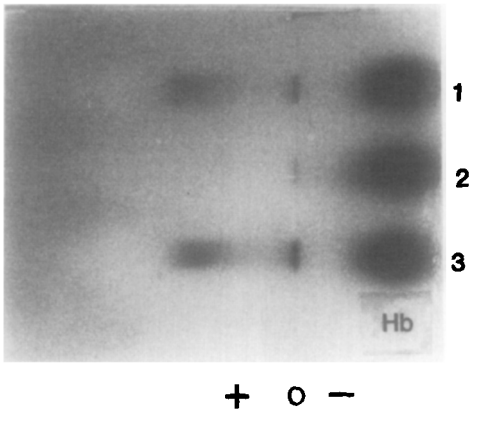
	mM Glucose Consumed per Liter RBC's/hour (Mean ± S.D.)	% Glucose via Pentose Shunt	Micromoles Glucose Consumed via Shunt per Liter RBC's/hour
Control (3)	1.5±0.6	8.9±3.3	116.6±1.7
Acetylphenyl-hydrazine (3)	2.1±0.2	42.4±1.6	902.0±93.6
P	N.S.	<0.001	<0.001

The percentage of glucose metabolized via the pentose shunt pathway was decreased slightly, although when calculated in terms of actual micromoles of glucose, there was a large increase in glucose metabolism via the shunt. Similar results are found with infected G6PD deficient red cells. The total glucose consumption appears to be less than in normal infected cells because of the lower parasitemia (and reduced growth rate) found in G6PD deficient red cells (2). When the glucose consumption data are extrapolated to 100% parasitemia, there is no difference between normal and G6PD deficient cells with respect to glucose metabolism.

The results with infected red cells stand in contrast to findings with uninfected red cells challenged with acetylphenylhydrazine (Table III). In these experiments, there is an insignificant increase in total glucose consumption, but there is a 5-fold increase in pentose shunt activity.

3. Demonstration of NADP dependent Glutamate dehydrogenase: Electrophoresis of lysates from infected but not control human erythrocytes (Fig. 2) shows a single component anodally at pH 7.0 in the starch gel support medium. The intensity of the stain is proportional to the parasitemia. No stain was precipitated if either NADP or glutamate or both were omitted from the staining solution.

4. Alpha-ketoglutarate content: The generation of NAD(P)H by glutamate dehydrogenase results in the production of alpha-ketoglutarate. If this enzyme is generating NADPH in infected erythrocytes, then increased amounts of alpha-ketoglutarate should also be produced. Indeed, infected red cells contained approximately 4 times more alpha-ketoglutarate than control cells (Fig. 3)



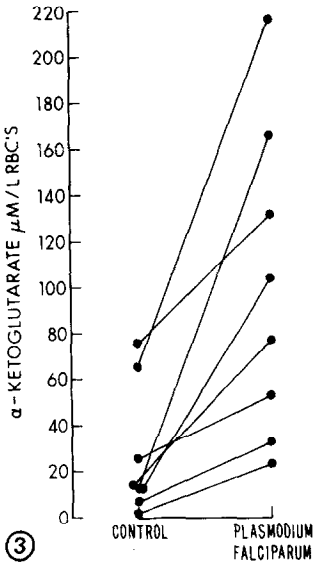


Figure 2: Starch gel electrophoresis, pH 7.0 of lysates from infected and control human red cells from the same donor. The gel was stained for the enzyme glutamate dehydrogenase using glutamate, NADP, phenazine methosulfate and MTT tetrazolium. The location of the enzyme is marked by a single anodal spot and is purple--the color of the formazan reaction product. Hb marks the migration of hemoglobin from the red cell lysates. Lane 1: P. falciparum infected RBC's, 12% parasitemia; lane 2: uninfected control; lane 3: P. falciparum infected RBC's, parasitemia 22%. Note that the intensity of the tetrazolium stain is approximately proportionate to the parasitemia.

Figure 3: Red cell alpha-ketoglutarate content of infected and control red cells maintained in culture medium for in vitro malaria growth. Blood from eight separate donors was used, and alpha-ketoglutarate was measured by the method of Bergmeyer and Bernt (12). There is a positive correlation between the increment in alpha-ketoglutarate content of infected cells and the degree of parasitemia ($r = +0.713$).

($p<0.01$). The increment in alpha-ketoglutarate was correlated with the parasitemia ($r = +0.73$).

DISCUSSION: The enhanced ability of normal red cells to maintain glutathione in the reduced form when infected with malaria suggests that the parasite actively participates in this process. This conclusion is further strengthened by the finding that omission of glucose abolishes the enhanced ability of infected cells to stabilize GSH. The decreased susceptibility of GSH in glucose-free infected red cells also suggests that despite the very different internal milieu of infected red cells (lower pH, the presence of degraded hemoglobin, etc.), acetylphenylhydrazine generates the usual oxidative stress. The increase in glucose utilization in infected red cells together with increased activity in

the pentose pathway are also consistent with the enhanced GSH stability which was noted in these cells.

Nevertheless, the pattern of glucose consumption in malaria does not suggest that the major cause of the increased glucose utilization is oxidative stress. As seen in the experiments with uninfected cells in the presence of acetylphenylhydrazine, an oxidative stress causes a diversion of glucose through the pentose shunt pathway with little or no increase in total glucose consumption. Clearly, parasitized red cells are utilizing more glucose for intense bursts of protein and nucleic acid synthesis and cell division--all activities which are not found in mature human erythrocytes. For these reasons, the data on glucose consumption and pentose shunt activity cannot be interpreted as a response to oxidative stress.

In normal, uninfected red cells, the only source of NADPH for reduction of oxidized glutathione is the pentose shunt pathway (4). These data suggest that malaria infected red cells may have at least one other pathway for generation of NADPH which could explain the enhanced stability of reduced glutathione. The enzyme glutamate dehydrogenase appears to be a parasite specific NADPH forming enzyme not found in uninfected human red cells. This enzyme has been described in other plasmodia species (13). The presence of increased amounts of alpha-ketoglutarate suggest that the enzyme is processing glutamate and generating large amounts of NADPH, but other sources of alpha-ketoglutarate such as formation by CO_2 fixation are also possible sources of this compound (14). The presence of another source of NADPH apart and separate from the pentose shunt pathway may render the G6PD deficient cell relatively secure in the presence of an oxidative stress since these cells generally contain above normal levels of glutathione reductase (4).

Finally, how do the findings described here relate to the malaria-G6PD hypothesis. Taken together, the data do not support the conclusion that an oxidative stress is limiting the growth of parasites in G6PD deficient cells. The G6PD deficient infected cells increase their total glucose consumption during infection, and there is even some increase in glucose consumed via the

pentose shunt. The infected normal cells do not appear unequivocally to be responding to any major oxidative stress, and GSH stability is enhanced, not decreased. Lastly, a possible pathway for the reduction of oxidized glutathione exists which is independent of the pentose shunt and may supply adequate NADPH for the needs of the cell.

If oxidative stress does not impair the growth of P. falciparum in G6PD deficient red cells, by what other mechanism could inhibition occur? The pentose pathway also generates ribosylpyrophosphate for nucleic acid metabolism (15), and may be the limiting factor for the parasites which are growing rapidly and need abundant precursors for RNA and DNA. These possibilities will require further investigation.

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