SYNERGISTIC ENHANCEMENT OF A COPPER CHELATOR, BATHOCUPROINE DISULPHONATE, AND CYSTEINE ON *IN VITRO* GROWTH OF *PLASMODIUM FALCIPARUM* IN GLUCOSE-6-PHOSPHATE DEHYDROGENASE-DEFICIENT ERYTHROCYTES

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Abstract. *In vitro* growth of *Plasmodium falciparum* is restricted in glucose-6-phosphate dehydrogenase (G6PD)-deficient erythrocytes (RBC), as a result of oxidative stress. Bathocuproine disulphonate (BCS), a copper chelator, as well as cysteine have been shown to synergistically stimulate the *in vitro* growth of various mammalian cells and *Trypanosoma* under oxygenated conditions. We examined the effects of these two chemicals on the *in vitro* growth of *P. falciparum* in G6PD-deficient RBC, and found that addition of BCS and cysteine synergistically enhanced the growth of the *P. falciparum* FCR-3 strain in these RBC to the same level as in normal RBC. However, BCS or cysteine alone had no stimulatory effect. To explain this synergistic enhancement, changes in thiol, NADPH and glutathione contents were investigated. After addition of cysteine alone, thiol content in the medium decreased rapidly, but when BCS was added, it was maintained at about 35% at 24 hours after incubation, suggesting that BCS stimulates parasite growth in G6PD-deficient RBC by inhibiting copper-mediated oxidation of cysteine in the medium. In these RBC, no increase in NADPH level, but a slight increase in glutathione, was observed in the presence of both BCS and cysteine. The slight increase of glutathione, was probably due to incorporation of cysteine from the medium, although this could not fully explain the synergistic growth enhancement. These findings taken together suggest that cysteine incorporated into G6PD-deficient RBC may help maintain the thiol groups in many proteins, such as membrane proteins, hemoglobin and enzymes, and plays an important role in maintaining an appropriate culture state necessary for parasite growth. We also examined the effects of BCS and cysteine on adaptation of wild isolates of *P. falciparum* to *in vitro* cultivation using the candle jar method. Although there was no drastic effect on growth enhancement, the presence of BCS and cysteine accelerated the appearance of schizonts in many isolates.

INTRODUCTION

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is an X-chromosome-linked hereditary abnormality with a high degree of clinical, biological and genetic variation. Evidence for a protective affect of G6PD deficiency against *Plasmodium falciparum* comes from both epidemiological associations (Ruwende *et al*, 1995; Myint-Oo *et al*, 1995) and *in vitro* studies (Roth *et al*, 1983a,b; Golenser *et al*, 1983; Miller *et al*, 1984). Results obtained by Usanga and Luzzatto (1985), however, suggest a gradual adaptation of the parasite by production of a

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parasite-encoded G6PD, and a recent field survey (Tantular *et al*, 1999) questions the protection of malaria infection by G6PD deficiency.

In G6PD-deficient erythrocytes (RBC), production of nicotinamide adenine dinucleotide phosphate (NADPH) is decreased and this may lead to a reduction of glutathione (GSH) levels (Roth *et al*, 1986a,b); its levels in G6PD-deficient RBC are reported to be a half of normal RBC (Miller *et al*, 1984; Bannai and Tateishi, 1988; Cappadoro *et al*, 1998). Consequently, an oxidative state generated in G6PD-deficient RBC has been proposed as an inhibitory mechanism of parasite growth in these RBC. Miller *et al* (1984) showed that pretreatment of cultures with the thiol-oxidizing agent, diamide, inhibited parasite growth in G6PD-deficient RBC but not in normal RBC. This study also indicated that an oxidative culture state might lead to growth inhibition in G6PD-deficient RBC. However, the precise suppression mechanism(s) of the *in vitro* growth of *P. falciparum* in G6PD-deficient RBC is still unclear (Barret, 1997).

In mammalian cells (Ishii and Bannai, 1985) and *Trypanosoma brucei* (Yabu *et al*, 1989), addition of a copper chelator, bathocuproine disulphonate (BCS), and cysteine synergistically enhance growth in culture by increasing thiol content. BCS is a nontoxic, copper-specific chelating agent and protects cysteine from oxidation to cystine in oxygenated conditions. In this paper, we hypothesize that the addition of BCS and cysteine might also enhance the growth of *P. falciparum* in G6PD-deficient RBC if growth inhibition is associated with an oxidative state resulting from decreased NADPH and GSH production. We further evaluated the effects of these chemicals on the adaptation of wild isolates of *P. falciparum* to standard *in vitro* culture condition. Here, we describe the synergistic enhancement of BCS and cysteine on the growth of culture-adapted *P. falciparum* in G6PD-deficient RBC, and discuss its stimulating mechanisms.

MATERIALS AND METHODS

Chemicals

Bathocuproine disulphonate (BCS) was purchased from Dojindo Laboratories (Kumamoto, Japan). Cysteine and 5, 5′-dithiobis (2-nitrobenzoic acid) (DTNB) were obtained from Wako Pure Chemicals (Osaka, Japan), and reduced NADPH and GSH from Sigma-Aldrich Co (Tokyo, Japan).

G6PD-deficient and normal RBC for *in vitro* culture

G6PD-deficient RBC was obtained from two healthy males with severe G6PD deficiency (Japanese and Indonesian), and used for experiments within 10 days after collected in acid-citrate-dextrose solution. The G6PD-deficient RBC from the Japanese donor (G6PD Beverly Hills type; Hirono *et al*, 1997) had 0 IU/g Hb, whereas those from the Indonesian donor had 0.13 IU/g Hb. Similarly, RBC with normal G6PD activity were obtained from two Japanese donors.

In vitro culture of *P. falciparum* (FCR-3 strain)

P. falciparum (FCR-3 strain) was cultured according to the candle jar method of Trager and Jensen (1976). The complete RPMI-1640 medium used in this study was supplemented with glutamine, 25 mM HEPES, 30 μg/ml gentamicin, 10% human serum and 1.0 mg/ml glucose. The cell suspensions were dispensed in 25-ml culture bottles with filter caps at 5% hematocrit, and cultivated at 37ºC in a candle jar. The supernatant was replaced daily with fresh complete medium. Thin smears were routinely stained with acridine orange (AO) method (Rothstein, 1958; Kawamoto, 1991) for parasite counts per 10,000 RBC and for morphological examination.

Treatment of BCS and cysteine and measurement of thiol content in culture medium

Stock solutions of BCS and cysteine were made every day in PBS at concentrations of 1 mM and 10 mM, respectively, and were diluted with complete medium just before experiments. Cysteine in PBS was reported to be stable (Ishii and Bannai, 1985).

To measure auto-oxidation of cysteine, 2 ml of the cell suspensions (5% hematocrit) in the complete medium containing 100 μM cys-

teine with or without BCS was incubated at 37° C in the candle jar. After incubation, thiol content in each culture medium was measured, following a method reported by Ishii and Bannai (1985), and compared to that in normal and G6PD-deficient RBC. Briefly, 1.2 ml of the medium was mixed with 1.2 ml of 0.2 M potassium phosphate buffer (pH 8.0) with 10 mM EDTA. The absorbance at 412 nm of the mixture was immediately measured, and then 0.1 ml of 10 mM DTNB was added. After 5 minute incubation with DTNB, the absorbance at 412 nm was again measured, and the thiol content was calculated from the difference between the absorbances, with cysteine in PBS as a standard.

Measurement of NADPH and GSH in G6PD-deficient RBC

NADPH production (*ie*, G6PD activity) in G6PD-deficient RBC was measured using a method reported by Fairbanks and Beutler (1962), with reduced NADPH as a standard. Changes in GSH concentration was also measured by a method reported by Beutler (1984). A standard curve using various concentrations of GSH was employed for the calculation of GSH content.

Collection of wild isolates of *P. falciparum*

This study was approved by the Ethical Review Committee of Nagoya University School of Medicine, the Myanmar Ministry of Health and the Health Department, Maumere, Flores Island, East Nusa Tenggara Province, Indonesia. Fresh isolates of *P. falciparum* were obtained between August 2000 and March 2002 at several locations in Myanmar and at Maumere (Win *et al*, 2001; Kawamoto *et al*, 2002). Informed consent was obtained from all symptomatic volunteers before diagnosis. Thin and thick smears were made from a single finger puncture, and examined using AO staining method (Rothstein, 1958; Kawamoto, 1991) at a magnification of x 400, using a light microscope and a halogen illuminator equipped with an interference filter for excitation of AO dye. If malaria-positive patients were diagnosed to have *P. falciparum* mono-infection with parasitemia above 0.5%, informed consent was obtained again, and 2-3 ml of venous blood was drawn into an evacuated blood collection tube with powdered sodium heparin.

Preservation and transportation of wild isolates in the complete medium in wet ice and cultivation by the candle jar method

Because of difficulties both in obtaining liquid nitrogen or dry ice and using facilities for *in vitro* cultivation in the field in Myanmar and Indonesia, we used a method for preservation of fresh isolates in wet ice (Nakazawa, 1985). Heparinized blood was kept in wet ice or with 'ice pack'. Within 7 hours after collection, 2 ml of heparinized blood was transferred using a syringe to a pre-cooled, 15-ml conical tube that contained 13 ml of the complete RPMI-1640 medium supplemented with 15% AB serum. These tubes were also preserved in wet ice or with 'ice pack', and were mixed gently once per day. Then they were transported to a laboratory in Surabaya, Indonesia. At the laboratory, cells were washed in complete RPMI-1640 medium with 15% serum three times, and cultivated by the candle jar method for 7-10 days. Isolates that grew well were maintained in culture for one month or more to establish culture-adapted isolates.

RESULTS

Synergistic enhancement of BCS and cysteine on *P. falciparum* growth in G6PD-deficient RBC

Development of *P. falciparum* FCR-3 strain in G6PD-deficient RBC and in normal RBC (control) was compared daily. As reported previously in many studies, parasite growth in G6PD-deficient RBC was suppressed to 50% when compared to control (Fig 1). Addition of both BCS (10 μM) and cysteine (100 μM), however, enhanced the growth in G6PD-deficient RBC to a similar level seen in the control, but no significant enhancement was observed in normal RBC. On the other hand, addition of each chemical alone did not enhance the growth in G6PD-deficient RBC; growth rate was the

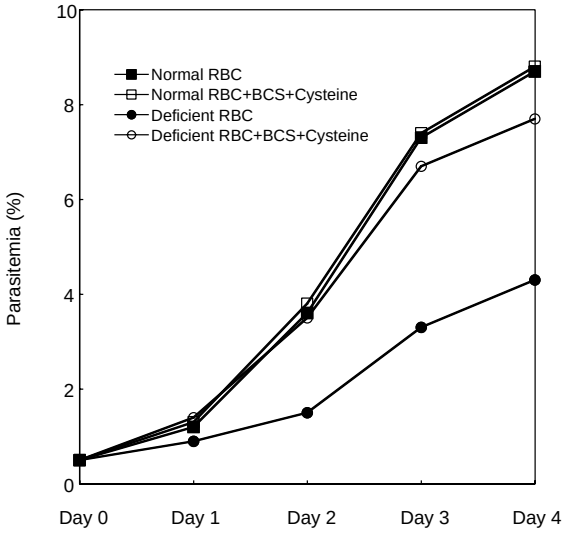

Fig 1–Effects of 10 μM BCS and 100 μM cysteine on the *Plasmodium falciparum* (FCR-3 strain) growth in G6PD-deficient RBC and in normal RBC. Addition of BCS and cysteine enhanced the growth in G6PD-deficient RBC. Data are expressed as the mean in three separate cultures. Significant difference ($p<0.01$) on day 2-4 was seen between cultures in G6PD-deficient RBC with and without both chemicals. This experiment was repeated using G6PD-deficient RBC from two donors three times each, and all data were similar as shown in this figure.

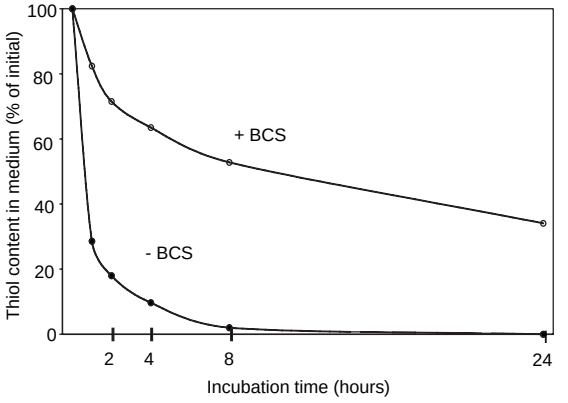

Fig 2–Inhibitory effects of BCS on the oxidation of cysteine in the medium cultivated with non-infected, G6PD-deficient RBC. Cysteine was added at 100 μM in the presence or absence of 10 μM BCS. Values represent the means of five determinations.

same with or without each chemical (data not shown). There was no significant difference in the growth patterns between G6PD-deficient RBC obtained from both donors. These results indicate that BCS and cysteine enhanced *P. falciparum* growth synergistically in G6PD-deficient RBC. Increased concentration of cysteine (200-300 μM) reduced parasite growth, and the optimum concentration was observed at 10 μM BCS and 100 μM cysteine (data not shown). Similar ranges of optimum concentrations have been reported in mammalian cells (Ishii and Bannai, 1985) and *Trypanosoma* (Yabu *et al*, 1989).

Thiol contents in the medium

In mammalian cells and *Trypanosoma*, the synergistic effect of BCS and cysteine was explained by the fact that BCS inhibits copper-mediated oxidation of cysteine in the culture media (Ishii and Bannai, 1985; Yabu *et al*, 1989). To explain the synergistic enhancement of *P. falciparum* growth in G6PD-deficient RBC, we also measured thiol contents in the culture medium after incubation with G6PD-deficient RBC. As reported by Ishii and Bannai (1985), the complete RPMI-1640 medium has no thiol.

Cysteine is unstable in culture media, and has a short half-life due to oxidation to cystine. Fig 2 shows the time course of oxidation of 100 μM cysteine in the complete medium with or without 10 μM BCS. As shown in Fig 2, oxidation of cysteine occurred rapidly without BCS, and within 1 hour, about 70% of cysteine was oxidized. In the presence of BCS, however, about 35% of cysteine were maintained in the medium after 24-hour incubation, as reported by Ishii and Bannai (1985) and Yabu *et al* (1989). In the presence of normal RBC, the same pattern of cysteine oxidation was observed as in Fig 2 (data not shown). These results all together may suggest that BCS enhances the *in vitro* growth of *P. falciparum* in G6PD-deficient RBC by inhibiting the oxidation of cysteine in the culture medium. At 20 μM of BCS, the thiol contents in the medium were the same level with those of 10 μM BCS (data not shown), indicating the inhibitory effect of BCS was maximal at about 10 μM. This concentration of BCS was

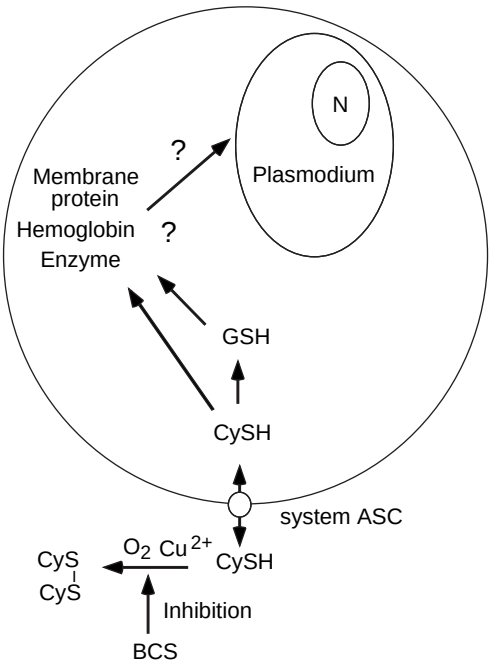

Fig 3–A schematic diagram of thiol supplying systems into G6PD-deficient RBC in the presence of BCS and cysteine, modified from Bannai (1984) and Bannai and Tateishi (1988).

similar to that giving maximal growth enhancement of *P. falciparum* in G6PD-deficient RBC.

Measurement of G6PD activity and GSH after addition of BCS and cysteine

G6PD-deficiency is caused by amino acid replacements in G6PD. Thus, it is unlikely that BCS and cysteine can activate the mutant enzyme. To ascertain this, we measured NADPH production in the G6PD-deficient RBC obtained from the Japanese donor, in the presence of BCS and cysteine. No increase in NADPH concentration was observed during cultivation for 24 hours, indicating that the synergistic enhancement by BCS and cysteine was not due to activation of the mutant G6PD.

As NADPH production was not seen in G6PD-deficient RBC in the presence of BCS and cysteine, it was expected that GSH level might be increased by reduction with incorporated cysteine and an enhancement of parasite growth might occur. When compared with GSH levels in the absence of BCS and cysteine, however, only a slight increase in GSH level (15± 3.6%; n=5) was observed in G6PD-deficient RBC after incubation for 24 hours. This increase could not fully explain the synergistic enhancement of growth at the same level seen in normal RBC.

Effects of BCS and cysteine on *in vitro* adaptation of wild isolates of *P. falciparum*

The candle jar method is the simplest method for cultivation of *P. falciparum* wild isolates, and it produces a gas condition of about 3% CO_2 and 17% O_2. This condition, however, is more oxidant than that found in the human host. So, we examined the hypothesis that an oxidative condition obtained by the candle jar method will be improved by addition of BCS and cysteine, and this may enable the establishment of culture-adapted isolates more easily by suppressing transformation to sexual stages in cultures.

A total of 14 wild isolates of *P. falciparum* preserved for 4-6 days were cultured, and growth was compared between cultures with or without BCS and cysteine (Table 1). All wild isolates could survive during preservation in wet ice for 4-6 days. On day 0, initial parasitemias in all isolates were maintained without a major loss, although it was difficult to assume whether all ring forms seen at the initiation of culture survived or not. On day 1, however, parasitemias decreased in many isolates, suggesting that a portion of ring forms were degenerated or damaged during preservation, as reported in *P. falciparum* culture-adapted clones by Nakazawa (1985).

On day 1, about half of wild isolates remained as ring forms without growth, then started growth thereafter. Among 14, two isolates (WW195 and WW206) transformed to young gametocytes in the presence of BCS and cysteine, and stopped asexual growth on day 4 and day 6, respectively. In another two isolates (WW133 and BH530), however, the parasites grew well in the presence of BCS and cysteine, whereas without both chemicals they transformed to sexual stages by day 6. In addition,

Table 1
Effects of BCS and cysteine on the *in vitro* adaptation of *Plasmodium falciparum* wild isolates.

Sample No.	Preserved day	BCS+ cysteine	Parasitemia on Day 0 (%)	Parasite stages observed[a]						
				Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
NK709	4	-	1.7	R	S>>>R	**R>T>S**	S>>R,T>YG	**R>T>S,YG**	T>S,R>YG	**R>>T,S,YG**[c]
		+		R	S>>>R	**R>T>S**	T>S>R>YG	**R,T>S,YG**	T>S>R,YG	**R>S>T,YG**[c]
NK722	4	-	2.1	R	T>S	**R>T,S>YG**	S>>R,T	R>T,S	S>>R,T,YG	**R>>T,S**[c]
		+		T>R	S>>>T	**R>>T,S>YG**	S>>T>R	T,R>S	S>>R,T,YG	**R>>T,S**[c]
KS316	5	-	3.4	R>T,S	R,T,S	T>R,S	R>T,S>YG	R,T,S>YG	**R,T,S>YG**	**R>>T,S,YG**[c]
		+		S>>T	R>>T,S	T>R>S	**R>>S,YG**	R,T,S>YG	R,T,S>YG	**R>>T,S,YG**[c]
PTM601	5	-	2.1	T>R	S>T,R	R>T>S	S>>R,T	**R>>T,S**	S>T>R	**R>T,S>YG**[c]
		+		T>R	S>>R,T	R,T>S	T>S>R,YG	**R>>T,S**	R,T,S	**R,T>S>YG**[c]
PTM627	5	-	2.2	S>T>R	**R>>S**	R,T,S,>YG	**R>T,S>YG**	R>T,S	**R>T,S>YG**	T>>R,S>YG[c]
		+		S>T>R	**R>>S**	R,T,S>YG	**R>T,S>YG**	T>S>R	**R,T,S>YG**	T>>R,S>YG[c]
PTM629	5	-	1.5	R	R,T>S	R,S>YG	**R>T>S**	R,T>YG	**R,T,S>YG**	T,S,R>YG
		+		R	S>>>T	R>S>YG	**R>T,S**	R,T>YG	**R,T,S>YG**	T,S,R>YG
PTM660	5	-	2.0	R	T	R,T>S	R>T,S	**R>>T,S**	R>S,T	**R,S>T,YG**[c]
		+		R	T	R,T>S	R>T,S	**R>>T,S**	T,S>R	**R,S>T>YG**[c]
PTM670	5	-	3.1	T>R	T>R>S	**R>>T,S**	T,S>R	**R>T,S>YG**	T>S>R,YG	S>>R,T,YG
		+		T>R	S>>R	**R>>T,S**	S>R>T	**R>T,S>YG**	T>S>R,YG	S>>R,T,YG
WW133	6	-	3.5	R>>T	R,T,S	T>>R>YG	T,S,YG	YG>>R	YG[b]	
		+		R>T	R,T,S	T>R>S,YG	R,T,S,YG	R,T,YG	**R,T,S,YG**	R,T,S,YG
WW195	6	-	1.4	R>T,S	R,T	YG>>R	YG[b]			
		+		R>T,S	R,T	R,T,S	R,T,S,YG	YG>>R	YG[b]	
WW206	6	-	3.5	R>>T	R,T	T,S	YG>>R	YG[b]		
		+		R>>T	R,T,S	T,S	T,S,YG	YG>>R	YG[b]	
BH530	6	-	2.5	R	R>>T	T>S	R>S	YG>>R	YG[b]	
		+		R	T>>R	S>>T	R,T,S	R,T,S,YG	R,T,YG	**R,T,S,YG**
BH555	6	-	2.0	R	T>S	R>S>T	R,T	**R>>T,S>YG**	R,T,S,YG	**R>>T, S,YG**[c]
		+		R	S>>T	R>>T,S	T>S>YG	**R>>T,S>YG**	R,T,S,YG	**R>>S>T,YG**[c]
BH557	6	-	1.5	R	S>T	R>S>T	S>>R>YG	**R,T,S>YG**	R,T>S,YG	**R>>T,S,YG**[c]
		+		R	S>>>T	**R>>S**	S>T,R,YG	**R>>T>S,YG**	R,T,S,YG	**R>>T,S,YG**[c]

[a]R=ring form; T=trophozoite; S=schizont; YG=young gametocyte. [b]All parasites transformed to young gametocytes. Gothic letters indicated that parasitemia reached to 4-5% with many ring forms, and they were sub-cultured or cryopreserved.[c]Isolates were continued cultivation for a month or more and established as culture-adapted isolates.

the asexual growth of eight isolates (NK722, KS316, PTM601, PTM629, PTM670, BH530, BH555 and BH557) was accelerated by the addition of both chemicals; the required time for developing to schizonts was faster in the presence of both chemicals than those without them. However, the appearance of sexual stage parasites was not suppressed in all isolates even in the presence of both chemicals.

DISCUSSION

In human RBC, cysteine in culture media is transported into the cells by two transporters, a major ASC system and a minor L system (Young *et al*, 1980; Bannai, 1984; Bannai and Tateishi, 1988; see Fig 3). Thiol groups derived from incorporated cysteine are protective to many proteins in RBC, such as hemoglobin, membrane proteins or enzymes, as well as utilized for GSH synthesis (Miller *et al*, 1984; Golenser *et al*, 1991; Scott *et al*, 1991). In the presence of BCS, cysteine might be incorporated into G6PD-deficient RBC, and thiols derived from incorporated cysteine may play important roles in the growth enhancement of *P. falciparum* in these cells (Fig 3). These findings are consistent with the evidence reported by Miller *et al* (1984) that G6PD-deficient RBC pretreated with the thiol-oxidizing agent, diamide, cannot support parasite growth. This was explained by a lack of thiol production in G6PD-deficient RBC after treatment with diamide, whereas in normal RBC thiol status is quickly recovered. Therefore, both studies may indicate an importance of sufficient thiols in RBC for parasite growth.

In the presence of BCS and cysteine, we expected an increase in GSH in G6PD-deficient RBC, that may produce a reduced culture state. However, only a slight increase in GSH was observed, and could not fully explain the synergistic enhancement of parasite growth in G6PD-deficient RBC. In this regard, Scott *et al* (1991) have reported that increases in intracellular GSH level do not protect hemoglobin oxidation in G6PD-deficient RBC, and concluded that decreased GSH concentration is not responsible for the increased oxidant sensitivity in G6PD-deficient RBC. In addition, recent studies (Mueller *et al*, 1997; Johnson *et al*, 2000) have also indicated that GSH-dependent peroxidase plays little or no role in the defense of RBC against exposure to peroxide. Overall, the enhancement by BCS and cysteine of parasite growth in G6PD-deficient RBC might be due to an appropriate redox culture state generated by thiol groups from incorporated cysteine (Fig 3).

It is well known that not all *P. falciparum* wild isolates can be adapted to standard *in vitro* culture (Thaithong and Beale, 1992). In unsuccessful attempts to culture, asexual parasites transform to sexual stages (young gametocytes) and stop asexual growth. Transformation to sexual stages (gametocytogenesis) in *P. falciparum* is known to be dependent upon both innate and environmental conditions (Carter and Miller, 1979), and many factors have been proposed to induce gametocytogenesis *in vitro*. Yet, the exact mechanisms still remain unclear (Lobo and Kumar, 1998; Dyer and Day, 2000). In the present study, the appearance of sexual stage parasites was not suppressed even in the presence of BCS and cysteine. However, our results may be suggestive of a slight stimulating effect of BCS and cysteine on the adaptation process of wild isolates to *in vitro* culture. To clarify this phenomenon, further investigation using more wild isolates should be conducted. It is worthy to note that *P. falciparum* wild isolates could be preserved in wet ice for at least 6 days.

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REFERENCES

Bannai S. Transport of cystine and cysteine in mammalian cells. *Biochem Biophys Acta* 1984; 779: 289-306.

Bannai S, Tateishi N. Maintenance of a glutathione level in mammalian cells. *Protein Nucleic Acid Enzyme* 1988; 33: 1442-9 (in Japanese).

Barret MP. The pentose phosphate pathway and parasitic protozoa. *Parasitol Today* 1997; 13: 11-6.

Beutler E. Red cell metabolism. A manual of biochemical methods. 3rd ed. Orlando, FL:Grune and Stratton, 1984: 131-5.

Cappadoro M, Giribaldi G, O'Brien E, *et al*. Early phagocytosis of glucose-6-phosphate dehydrogenase (G6PD)-deficient erythrocytes parasitized by *Plasmodium falciparum* may explain malaria protection in G6PD deficiency. *Blood* 1998; 92: 2527-34.

Carter R, Miller LH. Evidence for environmental modulation of gametocytogenesis in *Plasmodium falciparum* in continuous culture. *Bull WHO* 1979; 57: 37-52.

Dyer M, Day KP. Commitment to gametocytogenesis in *Plasmodium falciparum*. *Parasitol Today* 2000; 16: 102-7.

Fairbanks VF, Beutler E. A simple method for detection of erythrocyte glucose-6-phosphate dehydrogenase deficiency. *Blood* 1962; 20: 591-601.

Golenser J, Miller J, Spira DT, Novak T, Chevion M. Inhibitory effect of a Fava bean component on the *in vitro* development of *Plasmodium falciparum* in normal and glucose-6-phosphate dehydrogenase deficient RBC. *Blood* 1983; 61: 507-10.

Golenser J, Marva E, Chevion M. The survival of *Plasmodium* under oxidant stress. *Parasitol Today* 1991; 7: 142-6.

Hirono A, Fujii H, Takano T, Chiba Y, Azuno Y, Miwa S. Molecular analysis of eight biochemically unique glucose-6-phosphate dehydrogenase variants found in Japan. *Blood* 1997; 89: 4624-7.

Ishii T, Bannai S. The synergistic action of the copper chelator bathocuproine sulphonate and cysteine in enhancing growth of L1210 cells *in vitro*. *J Cell Physiol* 1985; 125: 151-5.

Johnson RM, Goyette G, Ravindranath Y, Ho YS. Red cells from glutathione peroxidase-1-deficient mice have nearly normal defenses against exogenous peroxides. *Blood* 2000; 96: 1985-8.

Kawamoto F. Rapid diagnosis of malaria by fluorescence microscopy using light microscope and interference filter. *Lancet* 1991; 337: 200-2.

Kawamoto F, Win TT, Mizuno S, *et al*. Unusual *Plasmodium malariae*-like parasites in Southeast Asia. *J Parasitol* 2002; 88: 350-7.

Lobo CA, Kumar N. Sexual differentiation and development in the malaria parasite. *Parasitol Today* 1998; 14: 146-50.

Miller J, Golenser J, Spira DT, Kosower NS. *Plasmodium falciparum:* thiol status and growth in normal and glucose-6-phosphate dehydrogenase deficient human RBC. *Exp Parasitol* 1984; 57: 239-47.

Mueller S, Riedet HD, Stremmel W. Direct evidence for catalase as the predominant H_2O_2-removing enzyme in human RBC. *Blood* 1997; 90: 4973-8.

Myint-Oo, Tin-Shwe, Marlaar-Than, O'Sullivan WJ. Genetic red cell disorders and severity of falciparum malaria in Myanmar. *Bull WHO* 1995; 73: 659-65.

Nakazawa S. Synchronization of cultured *Plasmodium falciparum* by cold treatment. *Kitakanto Med J* 1985; 35: 383-90 (in Japanese with English summary).

Roth EF Jr, Raventos-Suarez C, Rinaldi A, Nagel RL. The effect of X chromosome inactivation on the inhibition of *Plasmodium falciparum* malaria growth by glucose-6-phosphate-dehydrogenase-deficient red cells. *Blood* 1983a; 62: 866-8.

Roth EF Jr, Raventos-Suarez C, Rinaldi A, Nagel RL. Glucose-6-phosphate dehydrogenase inhibits *in vitro* growth of *Plasmodium falciparum*. *Proc Natl Acad Sci USA* 1983b; 80: 298-9.

Roth EF. Jr, Schulman S, Vanderberg J, Olson JA. Pathways for the reduction of oxidized glutathione in the *Plasmodium falciparum*-infected erythrocyte: can parasite enzyme replace host red cell glucose-6-phosphate dehydrogenase? *Blood* 1986a; 67: 827-30.

Roth EF Jr, Ruprecht RM, Schulman S, Vanderberg J, Olson JA. Ribose metabolism and nucleic acid

synthesis in normal and glucose-6-phosphate dehydrogenase-deficient human RBC infected with *Plasmodium falciparum*. *J Clin Invest* 1986b; 77: 1129-35.

Rothstein N. Vital staining of blood parasites with acridine orange. *J Parasitol* 1958: 588-94.

Ruwende C, Khoo SC, Snow RW, *et al*. Natural selection of hemi- and heterozygotes for G6PD deficiency in Africa by resistance to severe malaria. *Nature* 1995; 376: 246-9.

Scott MD, Zuo L, Lubin BH, Chiu DTY. NADPH, not glutathione, status modulates oxidant sensitivity in normal and glucose-6-phosphate dehydrogenase-deficient RBC. *Blood* 1991; 77: 2059-64.

Tantular IS, Iwai K, Lin K, *et al*. Field trials of a rapid test for G6PD deficiency in combination with a rapid diagnosis of malaria. *Trop Med Int Health* 1999; 4: 245-50.

Thaithong S, Beale G. Malaria parasites. Bangkok: Chulalongkorn University Research Report Series, 1992: pp 100.

Trager W, Jensen JB. Human malaria parasites in continuous culture. *Science* 1976; 193: 673-5.

Usanga EA, Luzzatto L. Adaptation of *Plasmodium falciparum* to glucose-6-phosphate dehydrogenase deficient host red cells by production of parasite-encoded enzyme *Nature* 1985; 313: 793-5.

Win TT, Tantular IS, Pusarawati S, *et al*. Detection of *Plasmodium ovale* by the ICT malaria P.f/P.v. immunochromatographic test. *Acta Tropica* 2001; 80: 283-4.

Yabu Y, Takayanagi T, Sato S. Long-term culture and cloning system for *Trypanosoma brucei gambiense* bloodstream forms in semi-defined medium *in vitro*. *Parasitol Res* 1989; 76: 93-7.

Young JD, Jones SEM, Ellory JC. Amino acid transport in human and in sheep RBC. *Proc R Soc London Ser* B 1980; 209: 355-75.