Regulation of intracellular glutathione levels in erythrocytes infected with chloroquine-sensitive and chloroquine-resistant *Plasmodium falciparum*

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Malaria is one of the most devastating tropical diseases despite the availability of numerous drugs acting against the protozoan parasite *Plasmodium* in its human host. However, the development of drug resistance renders most of the existing drugs useless. In the malaria parasite the tripeptide glutathione is not only involved in maintaining an adequate intracellular redox environment and protecting the cell against oxidative stress, but it has also been shown that it degrades non-polymerized ferriprotoporphyrin IX (FP IX) and is thus implicated in the development of chloroquine resistance. Glutathione levels in *Plasmodium*-infected red blood cells are regulated by glutathione synthesis, glutathione reduction and glutathione efflux. Therefore the effects of drugs that interfere with these metabolic processes were studied to establish possible differences in the regulation of the glutathione metabolism of a chloroquine-sensitive and a chloroquine-resistant strain of *Plasmodium falciparum*. Growth inhibition of *P. falciparum* 3D7 by D,L-buthionine-(S,R)sulphoximine (BSO), an inhibitor of γ -glutamylcysteine synthetase

(γ -GCS), and by Methylene Blue (MB), an inhibitor of glutathione reductase (GR), was significantly more pronounced than inhibition of *P. falciparum* Dd2 growth by these drugs. These results correlate with the higher levels of total glutathione in *P. falciparum* Dd2. Short-term incubations of Percoll-enriched trophozoite-infected red blood cells in the presence of BSO, MB and *N,N*′-bis(2-chloroethyl)-*N*-nitrosourea and subsequent determinations of γ -GCS activities, GR activities and glutathione disulphide efflux revealed that maintenance of intracellular glutathione in *P. falciparum* Dd2 is mainly dependent on glutathione synthesis whereas in *P. falciparum* 3D7 it is regulated via GR. Generally, *P. falciparum* Dd2 appears to be able to sustain its intracellular glutathione more efficiently than *P. falciparum* 3D7. In agreement with these findings is the differential susceptibility to oxidative stress of both parasite strains elicited by the glucose/glucose oxidase system.

Key words: drug resistance, oxidative stress.

INTRODUCTION

The tripeptide glutathione is the major low-molecular-mass thiol in almost all eukaryotes and has numerous functions in the cell. It acts as a redox buffer and the ratios of GSH to glutathione disulphide (GSSG) are usually between 10 and 100 [1,2]. The reduction of hydroperoxides by glutathione peroxidases results in the formation of GSSG that in turn is efficiently reduced by glutathione reductase (GR) to retain the crucial intracellular reducing environment. The removal of toxic metabolic by-products and the detoxification of xenobiotics by glutathione S-transferases are the major reactions that consume cellular glutathione. Glutathione conjugates are usually subjected to further catabolism or are excreted from the cell by GS-X pumps, and glutathione is lost during these reactions [3]. To sustain adequate glutathione levels, the tripeptide is synthesized by a distinct two-step biosynthetic pathway from the amino acids glutamate, cysteine and glycine. The enzymes responsible for glutathione synthesis are γ -glutamylcysteine synthetase (γ -GCS) and glutathione synthetase. Intracellular glutathione levels are mainly regulated by the reactions described above.

Plasmodium falciparum, the causative agent of malaria tropica, is responsible for 500 million clinical cases and two million deaths each year, particularly amongst young children [4]. The spread of drug resistance to chloroquine (CQ) and other drugs

available against the disease poses a serious health problem and emphasizes the need for the elucidation of the mechanisms of drug resistance and the discovery of modulators potentially reverting drug resistance [5]. CQ has been the drug of choice for the last 50 years not only because it used to be extremely effective but also because the treatment was affordable for the patients living in malaria-endemic areas. CQ prevents the polymerization of toxic ferriprotoporphyrin IX (FP IX) that is released as a result of haemoglobin digestion by the parasite. FP IX, if not polymerized, can damage the cell membranes and lead eventually to cell death.

Recently it has been shown that the transmembrane protein *P. falciparum* chloroquine-resistant transporter (*PfCRT*) located in the digestive vacuole of the malaria parasite is involved in the development of CQ resistance and that mutations of the protein can be correlated with such resistance [6,7]. Further it has been suggested that *P. falciparum* multidrug-resistance transporter (*PfMDR1*), a vacuolar transmembrane protein belonging to the family of ATP-binding-cassette transporters, has an impact on the resistance against CQ [8]. Both proteins are implicated in the accumulation of the drug in the parasite food vacuole, the site of CQ action. An additional mechanism involving glutathione has been proposed by Ginsburg and colleagues [9,10]. The tripeptide has been shown to degrade free FP IX and it was found that CQ inhibits this degradation process [9,10]. Glutathione levels in

Abbreviations used: BCIP, 5-bromo-4-chloroindol-3-yl phosphate; BCNU, *N,N*′-bis(2-chloroethyl)-*N*-nitrosourea; BSO, D,L-buthionine-(S,R)sulphoximine; CQ, chloroquine; CQR, CQ-resistant; CQS, CQ-sensitive; EBSS, Earle's balanced salt solution; FP IX, ferriprotoporphyrin IX; γ -GCS, γ -glutamylcysteine synthetase; GluOx, glucose oxidase; GR, glutathione reductase; GSSG, glutathione disulphide; MB, Methylene Blue; *PfCRT*, *P. falciparum* chloroquine-resistant transporter; *PfDd2*, *P. falciparum* strain Dd2; *Pf3D7*, *Plasmodium falciparum* strain 3D7; *PfMDR1*, *P. falciparum* multidrug-resistance transporter; RBC, red blood cell; SLO, streptolysin O; TRBC, trophozoite-infected red blood cell.

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CQ-resistant (CQR) and CQ-sensitive (CQS) strains of *Plasmodium berghei* correlate with the hypothesis that CQ prevents glutathione-mediated FP IX degradation and that drugs that alter the intracellular glutathione concentrations, such as D,L-buthionine-(S,R)sulphoximine (BSO), change the susceptibility of *Plasmodium* to CQ after administration [9–11]. Since *P. falciparum* synthesizes its own glutathione and also has an efficient GR, the parasite's intracellular glutathione concentration may be regulated via these metabolic pathways [12–15]. Therefore the present study was conducted using BSO to inhibit glutathione synthesis and Methylene Blue (MB) and/or *N,N*-bis(2-chloroethyl)-*N*-nitrosourea (BCNU) to inhibit GR in trophozoite-infected red blood cells (TRBCs). In addition, glutathione levels, glutathione efflux and the effect of the drugs on both enzymic activities were investigated in the CQS *P. falciparum* strain 3D7 (*Pf*3D7) and the CQR *P. falciparum* strain Dd2 (*Pf*Dd2). Further, the effects of the drugs on susceptibility to CQ and oxidative stress were determined.

EXPERIMENTAL

Materials

L-[U-¹⁴C]Glutamic acid (50 Ci/mmol) was purchased from Amersham-Buchler, Freiburg, Germany, and Albumax II was a product from Invitrogen. Hayem's reagent was purchased from Merck (Darmstadt, Germany). Streptolysin O (SLO) was a kind gift from Dr S. Bhakdi (University of Mainz, Mainz, Germany) [16]. Glucose oxidase (GluOx) from *Aspergillus niger*, 5-bromo-4-chloroindol-3-yl phosphate (BCIP) and Nitro Blue Tetrazolium were purchased from Sigma. All other chemicals were purchased from Sigma unless indicated otherwise.

Parasites

P. falciparum strains *Pf*Dd2 (originating from Indochina) and *Pf*3D7 (originating from The Netherlands) were maintained as described previously [14] with 5% human blood group A⁺ serum and 0.05% Albumax II. The infected cells were synchronized weekly by incubation of cells in 2 vol. of 0.3 M L-alanine/10 mM Hepes, pH 7.4, for 5 min at 37 °C according to [17]. For short-term incubations the cell culture was examined microscopically and harvested when the parasites were in the trophozoite stage. TRBCs were then concentrated to 95–100% parasitaemia by discontinuous Percoll/alanine gradient centrifugation [18]. The pellet was washed twice with Earle's balanced salt solution (EBSS) and subsequently cultivated at 10% haematocrit in the same medium for 2 or 3 h with or without addition of drugs. For determination of cell numbers, the cells were diluted 1:10 in Hayem's reagent and counted in a cell counter (Coulter Max M; Coulter Electronics, Krefeld, Germany). For compartment analysis, parasites were isolated from the red blood cells (RBCs) by using the pore-forming toxin SLO [19]. A total of 2×10^8 TRBCs were diluted in EBSS to a haematocrit of 10% and were incubated for 7 min with 1.43 µg of SLO at room temperature before being centrifuged at 1500 g for 5 min at 4 °C. The supernatants were used for the determination of either glutathione content, GR activities or γ-GCS activities, whereas the isolated parasites were washed twice in cold EBSS before further investigation.

Growth-inhibition assays

Drug testing was performed in duplicate in 24-well culture plates starting with synchronized parasites of 1–2% parasitaemia. Increasing concentrations of BSO (0–500 µM), MB (0–300 nM),

BCNU (0–200 µM) or GlcOx (20–50 m-units/ml) were added to the cultures with medium changes after 24 h. Parasite growth was examined microscopically by Giemsa-stained thin smears; the number of infected RBCs per 1000 RBCs was estimated. The IC₅₀ values were determined using GraphPad Prism version 3.0 (GraphPad Software).

Determination of glutathione level and GSSG efflux

Percoll-enriched *Pf*3D7 and *Pf*Dd2 were cultivated for 2 h in EBSS containing 5 mM glucose with or without addition of the respective IC₇₅ concentrations of BSO and MB. For determination of GSSG efflux, total glutathione of the culture medium was determined according to Tietze [20]. For determination of intracellular total glutathione, 1 vol. of Percoll-enriched TRBCs or isolated parasites were incubated with 2 vol. of 5% 5-sulphosalicylic acid, mixed and centrifuged for 15 min at 16000 g. The supernatant was used for the determination of total glutathione as described previously [14]. Briefly, the supernatant was added to 700 µl of 143 mM sodium phosphate (pH 7.5)/6.3 mM EDTA/0.34 mM NADPH, 100 µl of 6 mM 5,5'-dithiobis-(2-nitrobenzoate), and water was added to 950 µl. The mixture was incubated for 20 min at 20 °C and the reaction was started by adding 50 µl of GR (20 units · ml⁻¹). The concentration of glutathione was determined by observing the rate of change in absorption at 412 nm in a spectrophotometer (Uvikon 930; Kontron).

γ-GCS assay

Before the γ-GCS assay was carried out, the Percoll-enriched TRBCs were incubated for 3 h at 37 °C in EBSS containing 5 mM glucose with addition of the respective IC₇₅ concentration of BSO or BCNU. Subsequently, the cells were washed twice with cold EBSS. The cells were then divided into equal portions and one part was lysed with SLO to isolate the parasites. Parasite and TRBC pellets were then subjected to three cycles of freeze–thaw. The assay for γ-GCS was performed as described previously [14]. The 100 µl reaction contained 0.1 M Tris/HCl, pH 8.0, 20 mM MgCl₂, 2 mM EDTA, 1 mM [¹⁴C]glutamate (25 nCi), 10 mM L-α-aminobutyrate, 10 mM ATP, 5 mM creatine phosphate, 1 unit of creatine phosphokinase and the cell lysate of $(1.0\text{--}1.5) \times 10^8$ TRBCs or isolated *P. falciparum* cells. The cell lysate was supplemented with a protease inhibitor cocktail [4-(2-aminoethyl)benzenesulphonyl fluoride, pepstatin A, trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane ('E-64'), bestatin, leupeptin and apronitin] according to the manufacturer's recommendations (Sigma). Following incubation at 37 °C for 30 min, the reaction was terminated by the addition of 10 µl of 2 M HClO₄ and heating for 3 min at 95 °C. After cooling on ice, the samples were centrifuged at 16000 g for 5 min. The product of the enzyme reaction, γ-glutamylaminobutyrate, was determined by TLC using cellulose F plastic sheets (20 cm × 20 cm, 0.1 mm thickness; Merck) using a mobile phase of butan-1-ol/acetic acid/water (12:3:5, by vol.). γ-Glutamylaminobutyrate was revealed on the TLC sheets by autoradiography, the spots were cut out and the radioactivity was measured in 4 ml of Packard Pico Fluor 30 liquid scintillation cocktail.

GR assay

Percoll-enriched TRBCs were incubated for 3 h at 37 °C in EBSS containing 5 mM glucose with addition of the respective IC₇₅ concentration of BSO or BCNU. The TRBCs were divided into equal portions and one part was lysed with SLO to isolate the parasites from the RBCs. Subsequently both TRBCs and parasite

pellets were subjected to three cycles of freeze–thaw before 1 vol. of a butanol/chloroform solution (3:1.2, v/v) was added. The solution was thoroughly mixed before being centrifuged for 5 min at 16000 *g*. GR activity was determined from the upper phase as described previously [21–24].

Statistical analyses

Statistical analyses were performed using a two-tailed unpaired Student's *t* test ($P < 0.05$).

Preparation of RNA and Northern blotting

PfDd2 and *Pf3D7* highly synchronized trophozoite blood-stage forms (30–36 h) were incubated for 3 h with the IC_{75} of BCNU and saponin-lysed after incubation. For saponin lysis [25], TRBCs were incubated for 10 min at 4 °C in EBSS containing 0.15% saponin before adding 4 vol. of EBSS and incubating for 5 min at 37 °C. The released parasites were pelleted by centrifugation for 5 min at 3000 *g* and 4 °C. The pellet was washed twice with cold EBSS and resuspended in a 10-fold pellet volume of Trizol (Invitrogen) before freezing at –80 °C. Total RNA was extracted according to the manufacturer's instructions (Invitrogen). The blotting procedure was performed according to Kyes et al. [26]. Briefly, 30 µg of total RNA were separated by electrophoresis using a 1.4% agarose gel supplemented with 5 mM guanidine thiocyanate and subsequently transferred to a positively charged nylon membrane (Roche) with 7.5 mM NaOH as transfer buffer. The membrane was hybridized with a radio-labelled full-length γ -GCS cDNA probe [13] in 7% SDS/0.5 NaH₂PO₄, pH 7.2/2% dextran sulphate at 50 °C overnight. The membrane was washed three times in 75 mM NaCl/7.5 mM sodium citrate, pH 7.0/0.1% SDS for 10 min at 55 °C. The signals were visualized by exposure to a BioMax film (Kodak) overnight.

Protein preparation and protein blotting

SLO-isolated, highly synchronous trophozoites were diluted in PBS containing protease inhibitor cocktail (Sigma), sonified and centrifuged at 100000 *g*. Protein supernatant (7.5 µg) of *PfDd2* or *Pf3D7* was separated on a 7% SDS/polyacrylamide gel. The blotting procedure was performed according to standard procedures [27]. A polyclonal rabbit serum against recombinant *PfGR* was used as the first antibody [22] at a dilution of 1:10000. As a second antibody alkaline phosphatase-conjugated goat anti-rabbit IgG from Sigma was used (1:10000 dilution). The blot was developed using 33 µl of a 50 mg/ml stock solution of Nitro Blue Tetrazolium and 17 µl of a 50 mg/ml stock solution of BCIP mixed with 5 ml of alkaline phosphate substrate buffer (100 mM Tris/HCl/100 mM NaCl/5 mM MgCl₂, pH 9.5).

RESULTS

Effect of glutathione-modulating drugs on the survival and glutathione levels of *Pf3D7*- and *PfDd2*-infected TRBCs

In order to determine whether erythrocytic stages of *Pf3D7* and *PfDd2* are differentially affected by the glutathione-modulating drugs BSO, MB and BCNU, the IC_{50} values for growth inhibition *in vitro* were determined for both parasite strains. As shown in Figure 1, the IC_{50} values for BSO, MB and BCNU determined for *Pf3D7* were 26 µM, 12 nM and 33 µM, respectively, whereas for *PfDd2*, twice the amount of BSO (59 µM) and four times the MB concentration (51 nM) was required to obtain 50% inhibition of parasite growth. These differences are significant

Figure 1 Growth-inhibition curves of *PfDd2*- and *Pf3D7*-infected RBCs incubated with (A) BSO, (B) BCNU and (C) MB

The drug screening was performed for 48 h, as detailed in the Experimental section. Means $\pm$ S.D. from four or five independent determinations are shown. IC_{50} values were statistically analysed with an unpaired, two-tailed Student's *t* test ($P < 0.05$). *Significantly different IC_{50} values between strains on one graph.

(unpaired, two-tailed Student's *t* test, $P < 0.05$), indicating that TRBCs infected with *Pf3D7* are more susceptible to disruption of their glutathione metabolism than *PfDd2*-infected TRBCs. The effect of BCNU was similar for both strains. BCNU not only inhibits GR but also derivatizes reduced glutathione and leads to depletion of the tripeptide in TRBCs [28].

To further investigate whether the differential susceptibility of the parasites towards BSO and MB was due to differences in the glutathione levels in infected erythrocytes or in the parasites, these were determined before and after incubation with either BSO or MB (Table 1). Glutathione levels in TRBCs infected with either *Pf3D7* or *PfDd2* are not significantly different, although the glutathione content in *Pf3D7* TRBCs was consistently found to be 10% lower than in *PfDd2* TRBCs. Interestingly, the glutathione levels in the parasite compartments differed significantly, with *PfDd2* containing twice as much of the tripeptide

Table 1 Glutathione content for *PfDd2*- and *Pf3D7*-infected TRBCs and isolated parasites

The GSH content was determined after a 2 h incubation of Percoll-enriched TRBCs with the respective IC_{75} BSO concentration, IC_{75} MB concentration or without drug addition. For compartment analyses, the cells were subsequently lysed with SLO. *, Significant difference between strains ($P < 0.05$). The means $\pm$ S.D. from four or five independent determinations are shown. Glutathione contents of control TRBCs and parasites were normalized to 100%. The GSH levels in parentheses show percentages of treated TRBCs and parasites with reference to these basic GSH levels.

	Strain	Glutathione (nmol $\cdot 10^{10}$ cells $^{-1}$)	
		TRBCs	Parasite
Control	<i>PfDd2</i>	812.3 $\pm$ 58.0 (100%)	134.3 $\pm$ 15.6* (100%)
Control	<i>Pf3D7</i>	732.1 $\pm$ 168.5 (100%)	66.6 $\pm$ 9.8* (100%)
+ IC_{75} BSO _{Dd2}	<i>PfDd2</i>	614.6 $\pm$ 100.4 (76%)	111.0 $\pm$ 19.8* (82%)
+ IC_{75} BSO _{3D7}	<i>Pf3D7</i>	620.0 $\pm$ 140.6 (85%)	45.7 $\pm$ 16.8* (69%)
+ IC_{75} BSO _{Dd2}	<i>Pf3D7</i>	577.7 $\pm$ 130.2 (79%)	40.7 $\pm$ 11.7* (61%)
+ IC_{75} MB _{Dd2}	<i>PfDd2</i>	665.9 $\pm$ 98.7* (82%)	105.7 $\pm$ 14.1* (79%)
+ IC_{75} MB _{3D7}	<i>Pf3D7</i>	527.4 $\pm$ 93.6 (72%)	57.5 $\pm$ 22.3* (86%)
+ IC_{75} MB _{Dd2}	<i>Pf3D7</i>	413.0 $\pm$ 70.7* (56%)	46.8 $\pm$ 11.2* (70%)

Table 2 GR activities of *PfDd2*- and *Pf3D7*-infected TRBCs and isolated parasites

Percoll-enriched TRBCs were cultured for 3 h with the respective IC_{75} concentrations of BSO, with 92 μ M BCNU or without drug addition. For compartment analyses, the cells were subsequently lysed with SLO. One unit of GR activity is defined as the amount (in μ mol) of NADPH oxidized per min. The asterisks represent significant differences between strains ($P < 0.05$). The means $\pm$ S.D. from four or five independent determinations are shown. GR activities of untreated TRBCs and parasites were normalized to 100%. The GR activities in parentheses show percentages of treated TRBCs and parasites with reference to these basic activities.

	Strain	GR activity (m-units $\cdot 10^{10}$ cells $^{-1}$)	
		TRBCs	Parasite
Control	<i>PfDd2</i>	966.0 $\pm$ 181.6* (100%)	356.7 $\pm$ 106.8* (100%)
Control	<i>Pf3D7</i>	1446.0 $\pm$ 204.4* (100%)	774.7 $\pm$ 12.9* (100%)
+ IC_{75} BSO _{Dd2}	<i>PfDd2</i>	898.0 $\pm$ 112.6* (93%)	391.7 $\pm$ 132.7* (110%)
+ IC_{75} BSO _{3D7}	<i>Pf3D7</i>	1328.0 $\pm$ 180.9* (92%)	806.3 $\pm$ 40.6* (104%)
+ IC_{75} BSO _{Dd2}	<i>Pf3D7</i>	1352.0 $\pm$ 104.2* (93%)	762.0 $\pm$ 76.1* (98%)
+ IC_{75} BCNU	<i>PfDd2</i>	153.0 $\pm$ 17.5* (16%)	35.0 $\pm$ 18.4* (10%)
+ IC_{75} BCNU	<i>Pf3D7</i>	106.0 $\pm$ 13.2* (7%)	66.6 $\pm$ 2.4* (9%)

than *Pf3D7* (Table 1). This difference could be responsible for the distinct susceptibilities of the parasites to BSO and MB. Incubating the parasitized cells with BSO and MB for 2 h and subsequently determining their glutathione content further tested this hypothesis. For these short-term incubations we decided to apply concentrations of the respective drugs that led to a 75% growth reduction in the 48 h growth-inhibition assay.

Under these conditions, BSO and MB reduce the glutathione levels by 20–30% in TRBCs and parasite compartments of both strains. The results emphasize that a much higher concentration of both drugs is required to reduce the glutathione levels of *PfDd2*-infected TRBCs to the same extent as those of *Pf3D7* TRBCs (134 μ M versus 64 μ M BSO and 200 nM versus 40 nM MB). In order to be able to directly compare the effect of the inhibitors in both parasite strains, *Pf3D7* TRBCs and isolated parasites were also incubated with 134 μ M BSO and 200 nM MB (IC_{75} *PfDd2*) and subsequently their glutathione levels were determined (Table 1). As expected, the effect of both drugs on *Pf3D7* was more pronounced than on *PfDd2*. These results

Figure 2 Western blot analysis of *PfDd2* and *Pf3D7* parasite proteins

Highly synchronized trophozoites were isolated by SLO lysis, sonicated and centrifuged at 100 000 *g*. Then 7.5 μ g of the supernatant were used for the analysis and probed with a polyclonal rabbit antiserum against recombinant GR as described in the Experimental section. Molecular-mass markers (in kDa) from Bio-Rad are indicated on the left.

stress that there clearly is a difference in the effect that inhibition of γ -GCS by BSO has on CQR or CQS *P. falciparum*, which may be attributable to the relative abundance or the regulation of this enzyme in the parasites. Since MB is not entirely specific for the inhibition of GR, it remains unclear whether the different effects of the drug are indeed solely attributable to GR inhibition [29].

Intracellular glutathione levels in *P. falciparum*-infected TRBCs are mainly regulated by γ -GCS, GR and GSSG efflux. Therefore these three parameters were investigated further to elucidate their role for the control of glutathione levels in both parasite strains.

γ -GCS and GR activities

The differences in glutathione levels, BSO and MB susceptibilities observed in *Pf3D7* and *PfDd2* may be explained by differences in their GR or γ -GCS activities. In order to test this, the activities of both enzymes were determined in *Pf3D7*- and *PfDd2*-infected TRBCs. The GR activity of *Pf3D7* TRBCs is 1.5-fold higher than its equivalent in *PfDd2* TRBCs (Table 2). The addition of BSO to the culture hardly affects GR activities, but after incubation with the IC_{75} concentration of BCNU, the activities are decreased to 16% (*PfDd2* TRBCs) and 7% (*Pf3D7* TRBCs). Analysis of the parasite compartments revealed that GR activity in *Pf3D7* parasites is twice as high as in *PfDd2* parasites. BCNU treatment reduced GR activities in both parasite strains to approx. 10% of untreated cells. Nevertheless, the absolute GR activity remains twice as high in *Pf3D7* compared with *PfDd2* even after BCNU treatment (Table 2). To analyse whether different protein levels are the reason for the differences in GR activities in *Pf3D7* and *PfDd2*, a Western blot of parasite extracts was performed using *P. falciparum* GR antibodies. As shown in Figure 2, the GR protein levels in both parasite strains are comparable, indicating that different GR activities in TRBCs infected with *Pf3D7* or *PfDd2* are likely to be due to kinetic differences between the parasite enzymes. Previous results obtained with recombinant GRs from *Pf3D7* and *PfK1*, where the specific activity of recombinant *PfK1* GR was higher than

Table 3 γ -GCS activities of *PfDd2*- and *Pf3D7*-infected TRBCs and isolated parasites

Percoll-enriched TRBCs were cultured for 3 h with the respective IC_{75} concentrations of BSO or BCNU or without drug addition. For compartment analyses, the cells were subsequently lysed with SLO. One unit of γ -GCS activity is defined as the amount (in μ mol) of γ -glutamylaminobutyrate produced per min. The asterisks represent significant differences between strains ($P < 0.05$). Means $\pm$ S.D. are given from four to five independent experiments. γ -GCS activities of untreated TRBCs and parasites were determined and normalized to 100%. The data given in parentheses are with reference to these normalized γ -GCS activities.

		γ -GCS activity (m-units $\cdot 10^{10}$ cells $^{-1}$)	
Strain		TRBC	Parasite
Control	<i>PfDd2</i>	117.3 $\pm$ 4.5* (100%)	12.7 $\pm$ 0.5* (100%)
Control	<i>Pf3D7</i>	80.1 $\pm$ 5.0* (100%)	9.4 $\pm$ 1.8* (100%)
+ IC_{75} BSO _{Dd2}	<i>PfDd2</i>	44.2 $\pm$ 9.2* (38%)	10.7 $\pm$ 2.1* (84%)
+ IC_{75} BSO _{3D7}	<i>Pf3D7</i>	18.6 $\pm$ 5.8* (23%)	7.5 $\pm$ 0.6* (80%)
+ IC_{75} BSO _{Dd2}	<i>Pf3D7</i>	14.5 $\pm$ 1.1* (18%)	6.3 $\pm$ 1.6* (67%)
+ IC_{75} BCNU	<i>PfDd2</i>	119.8 $\pm$ 19.2* (102%)	18.8 $\pm$ 3.7 (148%)
+ IC_{75} BCNU	<i>Pf3D7</i>	85.7 $\pm$ 9.1* (107%)	13.1 $\pm$ 3.6 (139%)

that of *Pf3D7* GR, show that marked differences between GR activities of distinct *P. falciparum* strains are not unusual [22].

Comparison of the γ -GCS activities of *Pf3D7*- and *PfDd2*-infected TRBCs shows that they also differ significantly. γ -GCS activity in TRBCs infected with the CQR strain *PfDd2* is 36% higher than the activity measured in *Pf3D7*-infected TRBCs (Table 3). To investigate this difference in more detail, a compartment analysis was performed to reveal whether the differences in enzyme activities are more evident in the parasite compartment and whether the high γ -GCS activity of the erythrocyte covers subtle alterations within the parasite cell. The parasite compartment of both strains contains about 11% of the γ -GCS activity of the untreated TRBCs and there appears to be the same difference in γ -GCS activity, as found in whole TRBCs. In the presence of IC_{75} concentrations of BSO, γ -GCS activity is inhibited by 20% in the parasite compartment and the inhibition of enzyme activity is stronger in the erythrocytic compartment. This is very likely attributable to a relatively poor uptake of the drug into the parasite but could also indicate differences in the susceptibility of parasite- and host γ -GCS to BSO. Treating TRBCs infected with both parasite strains with the same concentration of BSO prior to determining γ -GCS activities, it becomes clear that the effect on *Pf3D7*-infected TRBCs as well as the parasites themselves is stronger than in *PfDd2* TRBCs and parasites, indicating that the parasites' glutathione synthesis is differentially affected by BSO. This effect correlates well with the differences in reduction of glutathione levels by BSO (see Table 1). The γ -GCS activity of *PfDd2* parasites is reduced by about 20% in the presence of 134 μ M BSO, and the glutathione content is reduced to the same extent. In the case of *Pf3D7*, γ -GCS activity is inhibited by 33%, and the parasites retain 61% of their glutathione. These results suggest that the glutathione synthesis in *Pf3D7* TRBCs is more susceptible to inhibition by BSO than γ -GCS activity in *PfDd2*-infected TRBCs, implying that the glutathione synthetic pathway is differentially regulated in the parasites.

Another interesting observation was that the depletion of intracellular glutathione levels by BCNU causes an increase of γ -GCS activity in the parasite compartment by about 50% (Table 3). In order to analyse whether this enhancement in γ -GCS activities is due to an increase in γ -GCS transcription, a Northern blot of BCNU treated and control parasites was performed.

Figure 3 Northern blotting

Synchronized trophozoites were incubated with 92 μ M BCNU (+) or left untreated (—) for 3 h and parasites were isolated by saponin lysis. The RNA was prepared from the parasite pellet. The RNA blot was probed with full-length γ -GCS [13]. 18 S RNA (bottom panel) was used as a reference.

Figure 3 shows that in both strains investigated the expected bands of 4.7 and 6.9 kb are present on the blot and that in BCNU-treated cells the signal is stronger than in untreated controls. These results suggest that γ -GCS can be up-regulated upon severe loss of glutathione caused by exogenous stresses.

Glutathione efflux in *PfDd2*- and *Pf3D7*-infected TRBCs

It has been shown previously that *P. falciparum*-infected erythrocytes lose a high amount of glutathione via a not-yet investigated/defined efflux mechanism [14,30]. In order to examine whether this efflux mechanism is responsible for the differential effects that BSO and MB have on *Pf3D7*- and *PfDd2*-infected erythrocytes, glutathione efflux, which according to Lüersen et al. [14] and Atamna and Ginsburg [30] essentially consists of GSSG, was determined for TRBCs infected with both parasite strains. As shown in Table 4 the efflux of both strains is comparable when not treated with either BSO or MB, despite the fact that TRBCs infected with *PfDd2* contain about 10% more glutathione than TRBCs infected with *Pf3D7*.

Inhibition of glutathione synthesis by BSO causes reduction of intracellular glutathione levels and as a response to BSO treatment GSSG efflux in *PfDd2* TRBCs is reduced by 20% whereas in *Pf3D7* TRBCs the efflux is only reduced by 10%. These data suggest that *Pf3D7* TRBCs are more susceptible to inhibition of glutathione synthesis, because their basic glutathione levels are lower, their glutathione synthesis rate is lower and they appear to be less able to adapt their GSSG efflux according to their needs than *PfDd2* TRBCs.

Table 4 Glutathione efflux from *PfDd2*- and *Pf3D7*-infected TRBCs

Percoll-enriched TRBCs were cultured for 2 h with the respective IC_{75} concentration of BSO or MB or without addition of drugs. Glutathione in the culture media was measured as described in the Experimental section. The means $\pm$ S.D. from four to five independent determinations are shown. Data in parentheses are with reference to control values (set at 100%).

	Glutathione (nmol $\cdot$ 10 ¹⁰ cells ⁻¹)	
	<i>PfDd2</i>	<i>Pf3D7</i>
Control	223.8 $\pm$ 44.1 (100%)	224.9 $\pm$ 13.5 (100%)
+ IC_{75} BSO	181.2 $\pm$ 32.2 (81%)	203.4 $\pm$ 10.2 (90%)
+ IC_{75} MB	293.0 $\pm$ 26.9 (131%)	262.5 $\pm$ 13.0 (117%)

Figure 4 Treatment with an exogenous oxidative stressor

Treatment of *PfDd2*- and *Pf3D7*-infected cells with GlcOx was performed in the absence or presence of 1 mM GSH. Means $\pm$ S.D. from four or five independent determinations are shown.

The inhibition of GR by MB results in an imbalance in the intracellular glutathione thiol redox status and both TRBCs respond with an enhanced efflux of GSSG to regulate their reducing environment. This adaptation appears to be more pronounced in *PfDd2* TRBCs than in *Pf3D7* TRBCs. However, as GR activity of *Pf3D7* TRBCs was determined to be higher than in *PfDd2* TRBCs, GSSG efflux may not be as important to maintain the intracellular redox state in this parasite strain.

In conclusion, the GSSG-efflux mechanism responds to the concentration of glutathione available in the cell and adjusts accordingly, e.g. after inhibition with BSO, efflux is reduced because less glutathione is available in the cell, and after treatment with MB glutathione efflux is increased to keep up an adequate ratio between GSH and GSSG.

Effect of oxidative stress and glutathione on the proliferation of *PfDd2* and *Pf3D7*

As the CQR strain *PfDd2* appears to be more resistant to glutathione-depleting stress than the CQS strain *Pf3D7*, it was of interest to analyse whether this difference is reflected by their general susceptibility to exogenously applied oxidative stress.

GlcOx is an enzyme that catalyses the production of D-gluconate and H_2O_2 from D-glucose and was used as a source for continuous oxidative stress. The effect of increasing concentrations of GlcOx on the parasites is shown in Figure 4. Growth inhibition of 50 % is reached at 20 m-units of GlcOx/ml for the CQS strain *Pf3D7*, and twice that concentration is required to inhibit *PfDd2* growth by 50 %. In order to analyse whether addition of 1 mM GSH was able to diminish the susceptibility towards GlcOx-induced oxidative stress, parasites were concomitantly incubated with GSH and GlcOx and their parasitaemia determined. It is shown in Figure 4 that GSH is indeed able to alter the inhibitory effect of GlcOx in both parasite strains but this appears to be more pronounced in *PfDd2*. These results are consistent with those obtained when applying other stresses that interfere with the glutathione levels of *Pf3D7*- and *PfDd2*-infected RBCs. They indicate a differential ability of these two parasite strains to respond to exogenous stress that interferes with their antioxidant mechanisms.

DISCUSSION

The development of CQ resistance in *Plasmodium* has been intensively investigated. A number of molecules responsible for alterations in CQ uptake, CQ extrusion or CQ binding to its ligand haem have been identified. Even though mutations in the vacuolar transmembrane protein *PfCRT* have been implicated to be responsible for the development of CQ resistance, other molecules such as *PfMDR1* and the tripeptide glutathione were shown to have an impact on CQ resistance in *P. falciparum* [6–9].

The toxicity of CQ is mainly a consequence of its binding to FP IX in the food vacuole of the parasite and preventing haem polymerization. The GSH-dependent degradation of FP IX plays an essential role in preventing fatal membrane damage in the parasitized cell [9]. It was shown that 3.5 mM BSO decreases the concentration of CQ required to kill *P. falciparum* *in vitro* [9,10] and there exists a correlation between CQ resistance and elevated glutathione levels of isolated *P. berghei* parasites [11,31]. Further it was shown that concomitant BSO and CQ application to mice infected with CQR *P. berghei* results in a drastic decrease of parasitaemia [11]. These observations imply that there exist marked differences in the intracellular glutathione levels of RBCs infected with CQR or CQS parasite strains. Using inhibitors that interfere with glutathione metabolism in *Plasmodium*-infected RBCs we aimed at identifying differences in the regulatory mechanisms that are responsible for maintaining glutathione levels in the CQS and CQR *P. falciparum* strains *Pf3D7* and *PfDd2*.

Indeed, BSO (inhibiting glutathione synthesis) and MB (inhibiting GR) both showed a significantly different IC_{50} for growth inhibition of both parasite strains, indicating that their glutathione metabolisms may be differentially regulated. Analyses of the glutathione levels of both TRBCs revealed that they only differ slightly, which is in contrast to the report by Dubois et al. [11] and Platel et al. [31], who found that the glutathione level in TRBCs infected with the *P. berghei* CQR parasites is up to three times higher than in TRBCs infected with CQS parasites. Detailed analyses of the glutathione level in the *Pf3D7* and *PfDd2* parasite compartments, however, revealed that it is indeed twice as high in the CQR parasites as in CQS parasites.

The distinct effects of BSO on the survival of *PfDd2* and *Pf3D7* may therefore be attributable to this difference in glutathione concentration in CQR and CQS parasites. Accordingly, the glutathione levels in both parasite strains are affected differentially when the same concentration of the drug was employed. BSO treatment resulted in a greater decrease of

Figure 5 Schematic overview of glutathione metabolism in *Pf3D7* and *PfDd2*

γ-GluCys, γ-glutamylcysteine.

glutathione in *Pf3D7*- than in *PfDd2*-infected TRBCs as well as in the parasites. This result is probably not only due to the higher basal levels of glutathione in *PfDd2* but also to the ability of *PfDd2*-infected TRBCs to adapt GSSG efflux more efficiently, as there is less GSSG efflux in BSO-treated *PfDd2* compared with *Pf3D7*. Further, γ-GCS activities in *PfDd2*-infected TRBCs and parasites are less affected by BSO than in *Pf3D7*-infected TRBCs and parasites. Nevertheless, both parasite strains are able to respond to exogenous stress, as incubation of TRBCs with BCNU results in up-regulation of γ-GCS mRNA levels and activity in *PfDd2* and *Pf3D7*. These results imply that *P. falciparum* (whether CQR or CQS) are able to adapt the transcription of genes encoding enzymes of the glutathione synthesis pathway according to their requirements.

The differential effect on the growth of the parasitized cells by MB appears not to be due to differences in the GR activities of both TRBCs. The concentrations required to inhibit 50 % growth of *Pf3D7* is 4-fold lower than the IC_{50} for *PfDd2*. Surprisingly this difference is not reflected by the GR activities determined in both parasite strains. *PfDd2*-infected TRBCs and parasites show lower GR activities than *Pf3D7*-infected TRBCs and parasites.

Therefore the impact of MB treatment on the viability of the parasites may be due to effects the drug has on other targets [29]. However, TRBCs infected with *Pf3D7* and *PfDd2* both showed increased GSSG efflux and decreased glutathione levels upon treatment with MB, confirming that the drug affects GR. Indeed, the effects of MB on GSSG efflux are distinct in both parasite strains and the results imply that TRBCs infected with *PfDd2* respond more efficiently to MB treatment by an increase of GSSG efflux upon inhibition of GR activity. In conclusion, our results show that *PfDd2*-infected TRBCs are able to preserve intracellular glutathione and their intracellular thiol redox environment more efficiently than *Pf3D7*-infected TRBCs under stress conditions.

Since the glutathione system represents one of the major antioxidant systems of the cell, we tested whether the susceptibility towards exogenous oxidative stress is different in TRBCs infected with either CQR or CQS parasites. It appeared that TRBCs infected with *PfDd2* are less susceptible to H_2O_2 than the CQS-parasite-infected TRBCs.

Taking our results into account we conclude that *PfDd2* and *Pf3D7* show marked differences in their glutathione metabolism. Figure 5 depicts the most important regulators of glutathione availability in the parasite and their role for the respective *P. falciparum* strain. The high GR activity enables *Pf3D7* to maintain their reducing environment efficiently and renders the parasites less dependent on regulation via their GSSG-efflux system. Therefore glutathione synthesis may be less important to sustain intracellular glutathione levels. In comparison, *PfDd2* appear to regulate their intracellular reducing environment by increased glutathione synthesis to compensate for the constant loss of GSSG, which is less effectively reduced by GR. The fact that *PfDd2* are able to sustain their intracellular glutathione concentration better may also render them less susceptible to inhibition of glutathione-dependent FP IX degradation by CQ and therefore more resistant to the drug.

This research was funded by the Deutsche Forschungsgemeinschaft (WA 395/12-1/2) and The Wellcome Trust (S. Müller is a Wellcome Senior Fellow). This work is part of a Ph.D. project performed at the University of Hamburg, Hamburg, Germany, by S. Meierjohann.

REFERENCES

- Anderson, M. E. (1985) Determination of glutathione and glutathione disulfide in biological samples. *Methods Enzymol.* **113**, 548–555
- Sies, H. (1989) Biochemistry of thiol groups: the role of glutathione. *Naturwissenschaften* **76**, 57–64
- Ishikawa, T., Li, Z. S., Lu, Y. P. and Rea, P. A. (1997) The GS-X pump in plant, yeast, and animal cells: structure, function, and gene expression. *Biosci. Rep.* **17**, 189–207
- World Health Organisation, Malaria Unit (2000) Global malaria control. *Bull. WHO* **71**, 281–284
- Kyle, D. E., Milhous, W. K. and Rossan, R. N. (1993) Reversal of *Plasmodium falciparum* resistance to chloroquine in Panamanian Aotus monkeys. *Am. J. Trop. Med. Hyg.* **48**, 126–133
- Fidock, D. A., Nomura, T., Talley, A. K., Cooper, R. A., Dzekunov, S. M., Ferdig, M. T., Ursos, L. M., Sidhu, A. B., Naude, B., Deitsch, K. W. et al. (2000) Mutations in the *P. falciparum* digestive vacuole transmembrane protein *PF3D7_1303* and evidence for their role in chloroquine resistance. *Mol. Cell* **6**, 861–871
- Cooper, R. A., Ferdig, M. T., Su, X. Z., Ursos, L. M., Mu, J., Nomura, T., Fujioka, H., Fidock, D. A., Roepe, P. D. and Wellems, T. E. (2002) Alternative mutations at position 76 of the vacuolar transmembrane protein *PF3D7_1303* are associated with chloroquine resistance and unique stereospecific quinine and quinidine responses in *Plasmodium falciparum*. *Mol. Pharmacol.* **61**, 35–42
- Reed, M. B., Saliba, K. J., Caruana, S. R., Kirk, K. and Cowman, A. F. (2000) Pgh1 modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum*. *Nature (London)* **403**, 906–909
- Ginsburg, H., Famin, O., Zhang, J. and Krugliak, M. (1998) Inhibition of glutathione-dependent degradation of heme by chloroquine and amodiaquine as a possible basis for their antimalarial mode of action. *Biochem. Pharmacol.* **56**, 1305–1313

- 10 Famin, O., Krugliak, M. and Ginsburg, H. (1999) Kinetics of inhibition of glutathione-mediated degradation of ferriprotoporphyrin IX by antimalarial drugs. *Biochem. Pharmacol.* **58**, 59–68
- 11 Dubois, V. L., Platel, D. F., Pauly, G. and Tribouley-Duret, J. (1995) *Plasmodium berghei*: implication of intracellular glutathione and its related enzyme in chloroquine resistance *in vivo*. *Exp. Parasitol.* **81**, 117–124
- 12 Färber, P. M., Arscott, L. D., Williams, Jr, C. H., Becker, K. and Schirmer, R. H. (1998) Recombinant *Plasmodium falciparum* glutathione reductase is inhibited by the antimalarial dye methylene blue. *FEBS Lett.* **422**, 311–314
- 13 Lüersen, K., Walter, R. D. and Müller, S. (1999) The putative gamma-glutamylcysteine synthetase from *Plasmodium falciparum* contains large insertions and a variable tandem repeat. *Mol. Biochem. Parasitol.* **98**, 131–142
- 14 Lüersen, K., Walter, R. D. and Müller, S. (2000) *Plasmodium falciparum*-infected red blood cells depend on a functional glutathione *de novo* synthesis attributable to an enhanced loss of glutathione. *Biochem. J.* **346**, 545–552
- 15 Meierjohann, S., Walter, R. D. and Müller, S. (2002) Glutathione synthetase from *Plasmodium falciparum*. *Biochem. J.* **363**, 833–838
- 16 Bhakdi, S. and Tränkle, J. (1985) Complement activation and attack on autologous cell membranes induced by streptolysin-O. *Infect. Immun.* **48**, 713–719
- 17 Braun Breton, C., Rosenberry, T. L. and Pereira da Silva, L. H. (1990) Glycolipid anchorage of *Plasmodium falciparum* surface antigens. *Res. Immunol.* **141**, 743–755
- 18 Kanaani, J. and Ginsburg, H. (1989) Metabolic interconnection between the human malarial parasite *Plasmodium falciparum* and its host erythrocyte. Regulation of ATP levels by means of an adenylate translocator and adenylate kinase. *J. Biol. Chem.* **264**, 3194–3199
- 19 Burghaus, P. A. and Lingelbach, K. (2001) Luciferase, when fused to an N-terminal signal peptide, is secreted from transfected *Plasmodium falciparum* and transported to the cytosol of infected erythrocytes. *J. Biol. Chem.* **276**, 26838–26845
- 20 Tietze, F. (1969) Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. *Anal. Biochem.* **27**, 502–522
- 21 Krohne-Ehrich, G., Schirmer, R. H. and Untucht-Grau, R. (1977) Glutathione reductase from human erythrocytes. Isolation of the enzyme and sequence analysis of the redox-active peptide. *Eur. J. Biochem.* **80**, 65–71
- 22 Gilberger, T. W., Schirmer, R. H., Walter, R. D. and Müller, S. (2000) Deletion of the parasite-specific insertions and mutation of the catalytic triad in glutathione reductase from chloroquine-sensitive *Plasmodium falciparum* 3D7. *Mol. Biochem. Parasitol.* **107**, 169–179
- 23 Krauth-Siegel, R. L., Müller, J. G., Lottspeich, F. and Schirmer, R. H. (1996) Glutathione reductase and glutamate dehydrogenase of *Plasmodium falciparum*, the causative agent of tropical malaria. *Eur. J. Biochem.* **235**, 345–350
- 24 Worthington, D. J. and Rosemeyer, M. A. (1976) Glutathione reductase from human erythrocytes. Catalytic properties and aggregation. *Eur. J. Biochem.* **67**, 231–238
- 25 Christophers, S. R. and Fulton, J. D. (1939) Experiments with isolated malaria parasites (*Pl. knowlesi*) free from red cells. *Ann. Trop. Med. Parasitol.* **33**, 161–171
- 26 Kyes, S., Pinches, R. and Newbold, C. (2000) A simple RNA analysis method shows var and rif multigene family expression patterns in *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* **105**, 311–315
- 27 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) in *Molecular Cloning: a Laboratory Manual*, 2nd edn., Cold Spring Harbor Press, Cold Spring Harbor, NY
- 28 Stahl, W., Krauth-Siegel, R. L., Schirmer, R. H. and Eisenbrand, G. (1987) A method to determine the carbamoylating potential of 1-(2-chloroethyl)-1-nitrosoureas. *IARC Sci. Publ.* **84**, 191–193
- 29 Grellier, P., Sarlauskas, J., Anusevicius, Z., Marozienė, A., Houe-Levin, C., Schrevel, J. and Cenas, N. (2001) Antiplasmodial activity of nitroaromatic and quinoidal compounds: redox potential vs. inhibition of erythrocyte glutathione reductase. *Arch. Biochem. Biophys.* **393**, 199–206
- 30 Atamna, H. and Ginsburg, H. (1997) The malaria parasite supplies glutathione to its host cell—investigation of glutathione transport and metabolism in human erythrocytes infected with *Plasmodium falciparum*. *Eur. J. Biochem.* **250**, 670–679
- 31 Platel, D. F., Mangou, F. and Tribouley-Duret, J. (1999) Role of glutathione in the detoxification of ferriprotoporphyrin IX in chloroquine resistant *Plasmodium berghei*. *Mol. Biochem. Parasitol.* **98**, 215–223

Received 24 June 2002/3 September 2002; accepted 12 September 2002

Published as BJ Immediate Publication 12 September 2002, DOI 10.1042/BJ20020962