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GLUTATHIONE REDUCTASE-DEFICIENT ERYTHROCYTES AS HOST CELLS OF MALARIAL PARASITES

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Abstract—BCNU [1,3-bis(2-chloroethyl)-1-nitrosourea] and its less toxic derivative HeCNU [1-(2-chloroethyl)-3-(2-hydroxyethyl)-1-nitrosourea] are clinically-used antitumour drugs. In erythrocytes BCNU is a highly specific inhibitor of the enzyme glutathione reductase [H. Frischer and T. Ahmad, *J. Lab. clin. Med.* **89**, 1080 (1977)]. When treating erythrocytes *in vitro*, 50% enzyme inhibition was obtained with 1 μM BCNU or 3 μM HeCNU within 2 hr. The two drugs were used for preparing red cell populations with various levels of glutathione reductase activity; complete inhibition (≥98%) was only achieved when the medium contained glucose as a source of reducing equivalents. The erythrocytes were then tested in drug-free media as host cells for the malaria parasite *Plasmodium falciparum*. In the range of 0–300 mU/ml cells, there was a correlation between glutathione reductase activity and parasite growth; erythrocytes with an activity of less than 20 mU/ml did not serve as host cells for *P. falciparum* at all although these erythrocytes were viable. When the culture medium was supplemented with 20 mM glutathione (GSH), parasite growth was normal irrespective of the glutathione reductase level in the erythrocytes. This is consistent with the finding that poisoning glutathione reductase led to a 10-fold decrease of the cytosolic GSH level. Our results corroborate the concept that intraerythrocytic inhibition of glutathione reductase mimicks the biochemistry of drug-sensitive glucose-6-phosphate dehydrogenase deficiency (favism), an inherited condition which confers protection from malaria.

Recent studies [1, 2] have confirmed the hypothesis that favism (drug-sensitive glucose-6-phosphate dehydrogenase deficiency) confers resistance to malaria [3], and suggested to us that the biochemistry of favism might be mimicked by inhibiting the enzyme glutathione reductase in human erythrocytes [4]. This concept is consistent with the observation that a patient with hereditary GR deficiency developed the typical clinical picture of favism after the ingestion of fava beans [5]. 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU)‡ is a highly specific inhibitor of GR in erythrocytes [6]. Here we report on human erythrocytes which have been made GR-deficient by treating them with BCNU [6–9] or with HeCNU [10, 11]. The GR-deficient cells were tested as host cells of the malaria parasite *Plasmodium falciparum*. In the accompanying paper [12] a complementary aspect, the lack of parasite growth *in the presence* of the inhibitors is described. For the appropriate use of BCNU and HeCNU the following properties of glutathione reductase [13] must be accounted for.

The enzyme catalyzes the reaction $NADPH + H^+ + GSSG \rightleftharpoons NADP^+ + 2GSH$ [14]. During catalysis it changes between the two stable forms E and EH_2 both occurring in the cell [8, 15]:

$$E + NADPH + H^+ \rightarrow EH_2 + NADP^+$$
$$EH_2 + GSSG \rightarrow E + 2GSH.$$

E is characterized by the active site disulfide Cys58-Cys63 whereas in EH_2 these residues contain free SH-groups [16, 17]. It is the thiol of Cys58 which is modified by nitrosourea derivatives [11, 13, 18]. This explains why EH_2 can be poisoned whereas the oxidized form E is resistant to thiol reagents.

MATERIALS AND METHODS

Reagents and media

These were the same as in Ref. 12.

Parasite cultures and analytical methods

The following procedures were carried out as described in the accompanying paper [12]: cultivation and isolation of *Plasmodium falciparum*; glutathione reductase assay; protein determination; measurement of glutathione levels in erythrocytes.

Poisoning of glutathione reductase with nitrosoureas

(a) Non-parasitized red blood cells. Fresh erythrocytes from A (+) blood were washed three times with RPMI medium, taken up in this medium to give a hematocrit of 5% and then exposed to various

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‡ Abbreviations: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; GSH, reduced glutathione; GSSG, glutathione disulfide; HeCNU, 1-(2-chloroethyl)-3-(2-hydroxyethyl)-1-nitrosourea; GR, glutathione reductase (EC 1.6.4.2); PBS, phosphate buffered saline.

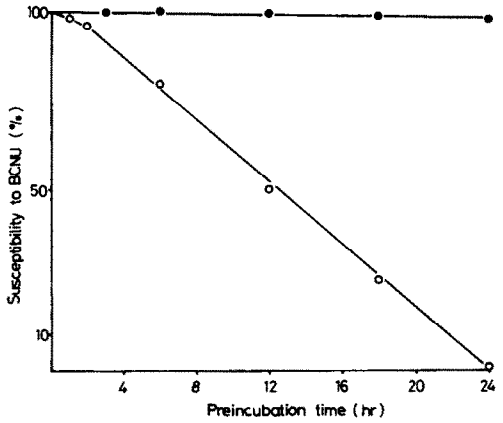

Fig. 1. Effect of glucose on the susceptibility of intraerythrocytic glutathione reductase to BCNU. The details are given in the experimental section. In the presence of glucose (4 mg/ml PBS buffer) glutathione reductase is susceptible to BCNU for at least 24 hr (●); without added glucose the enzyme becomes resistant to BCNU (○). The susceptibility to BCNU is expressed as the ratio of GR activity in a BCNU-treated sample divided by the GR activity of a matched control which had not been exposed to BCNU. The control samples at all time intervals contained approx. 1U GR/ml cells.

concentrations of BCNU or HeCNU at 37°. After pelleting and washing the cells 20 μl aliquots were lysed [12, 19] and assayed for glutathione reductase. The erythrocyte populations differing in GR activity—the range being 20 mU/ml to 1 U/ml—were tested as host cells for *P. falciparum*.

(b) Parasitized red blood cells. Erythrocytes with a parasitemia of 15% were treated with BCNU or HeCNU as described above. Then each sample was divided into two portions; one was lysed immediately and tested for GR activity. From the other portion the parasites were isolated, lysed and then assayed for GR.

Influence of added glucose on the effect of BCNU

Twenty-four ml red cells were portioned in two groups of 1 ml-samples. The samples of group I were incubated at 37° and pH 7.2 in 1 vol phosphate buffered saline (PBS) and the samples of group II in PBS containing 4 mg glucose/ml. Every 4 hours the samples were centrifuged and fresh PBS or glucose-containing PBS was added. At 0, 2, 4, 6, 12 and 24 hr, respectively, 1 sample of each group was treated with 250 μM BCNU for 1 hr at 37° prior to analysis for residual glutathione reductase activity. Each sample was compared with a matched control which had not been treated with BCNU.

RESULTS

Poisoning of intraerythrocytic glutathione reductase

GR can be poisoned by BCNU and related compounds but the proportion of susceptible enzyme is still a matter of controversy [6, 7, 20–22]. The results shown in Fig. 1 might help to explain the discrepancies. In erythrocytes, GR can be inhibited quantitatively if the glucose supply is sufficient for keeping the enzyme in the EH_2 form; as detailed above only this form is susceptible to BCNU. In the absence of glucose the predominant forms of glutathione reductase seem to be E or reversibly modified EH_2 both of which are resistant against BCNU action. One may conclude that an intact redox metabolism is a prerequisite for the quantitative inhibition of intracellular glutathione reductase by nitrosoureas (Figs 1–3).

As shown in Figs 2 and 3 GR is less reactive with HeCNU than with BCNU. Figure 3 demonstrates the effects of BCNU and HeCNU on different glutathione reductase species. For the erythrocyte enzyme and the two enzyme species present in parasitized erythrocytes [24, 25] the IC_{50} at 2 hr was determined to be 1 μM in the case of BCNU, and 4 μM in the case of HeCNU. When analyzing isolated parasites, the IC_{50} value appeared to be greater, and

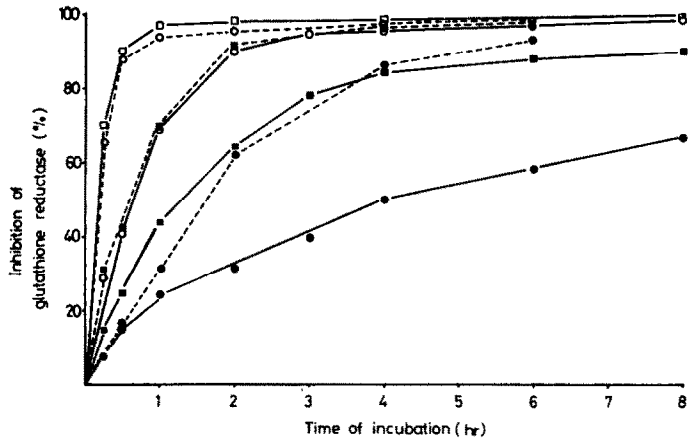

Fig. 2. Inhibition of erythrocyte GR with BCNU and HeCNU as a function of time. Freshly-drawn erythrocytes from healthy donors were washed three times with 10 vol. of RPMI medium and then taken up in this medium to give a hematocrit of 5%. The cells were incubated at 37° with BCNU (——) at concentrations of 1 μM (●), 3 μM (■), 10 μM (○), 30 μM (□), and with HeCNU (---) at 10 μM (●), 30 μM (■) and 100 μM (○). After the indicated intervals residual GR activity was determined in the lysates.

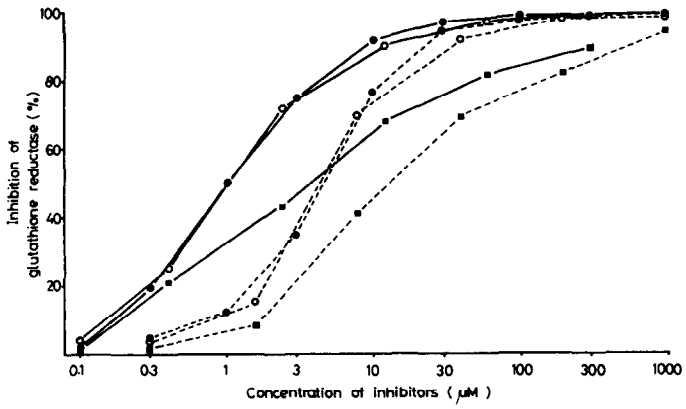

Fig. 3. Poisoning of glutathione reductase as a function of inhibitor concentration in the medium. Normal and parasitized erythrocytes were washed and suspended in RPMI medium as described in Fig. 2 and then incubated for 2 hr with various concentrations of BCNU and HeCNU, respectively. Incubation with BCNU (——), incubation with HeCNU (---), normal erythrocytes (●), parasitized erythrocytes (○), parasites isolated from parasitized erythrocytes (■).

more than 90% inhibition was not observed even when the parasitized red cells had been exposed to 300 μM for 2 hr. Studies on isolated GR from *P. falciparum* [24] will show whether the apparent low susceptibility to nitrosoureas is an intrinsic property of this enzyme or whether it is due to pharmacokinetic processes.

Erythrocytes with different levels of glutathione reductase as host cells of P. falciparum

For these experiments GR in red blood cells was poisoned to various degrees (from 30 to 98% using BCNU or HeCNU (Figs 4 and 5).Twenty mU/ml erythrocytes was the lowest level of the enzyme which could be measured reliably. This corresponds to 2% residual activity or 98% inhibition. Whatever the degree of inhibition, the residual GR activity in the erythrocytes was found to be constant for at least 72 hr under the conditions used for culturing *P. falciparum*. Even with an activity of less than 20 mU/ml cells, no haemolysis was observed. The GR deficient erythrocytes and untreated cells containing 1000 mU GR/ml were used for culturing *P. falciparum*. After 48 hr corresponding to 1 multiplication cycle of synchronized *P. falciparum* the erythrocyte populations with 100 mU GR/ml contained 50% less parasites than the control; no parasite growth was observed in erythrocytes with 20 mU/ml or less (Figs 4 and 5). This result indicates that parasite growth is correlated with the GR-content of the host erythro-

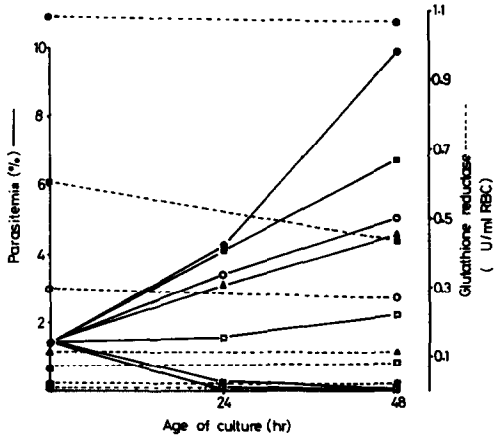

Fig. 4. Parasite growth in erythrocytes with different GR activities. Red cells with partially or totally poisoned glutathione reductase were prepared by treatment with BCNU (Fig. 3). Inoculation and parasite growth took place in the absence of BCNU. The GR activities per ml cells were: 1000 mU (cells without BCNU pretreatment) (●), 600 mU (■), 300 mU (○), 100 mU (▲), 40 mU (□), 20 mU (◨) and less than 20 mU (◑). Each point represents the average of three experiments. Parasitemia (——), glutathione reductase activity (---).

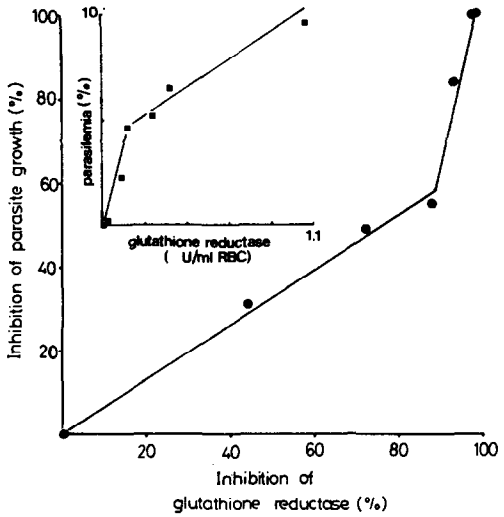

Fig. 5. Impairment of parasite growth in GR-deficient erythrocytes. The data for $t = 48$ hr were taken from Fig. 4. These data were also used for the insert which shows the parasitemia as a function of the erythrocyte GR activity present at $t = 0$ hr, that is prior to infection with *P. falciparum*.

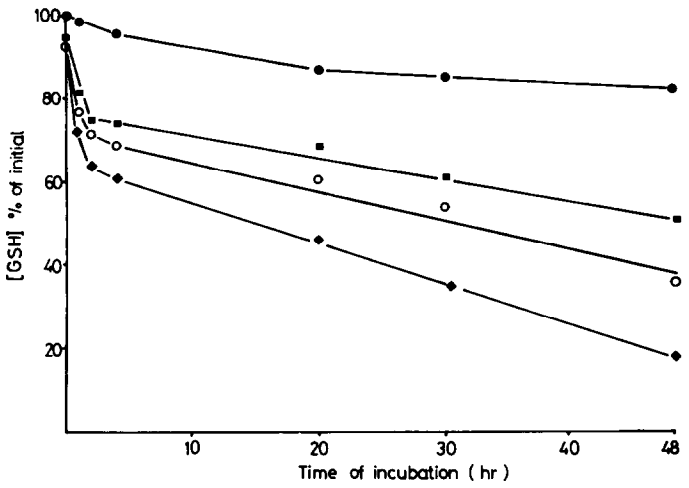

Fig. 6. Decrease of glutathione in red blood cells after BCNU-mediated inactivation of glutathione reductase. Erythrocytes were treated with BCNU for 1 hr as described in Fig. 3. The BCNU concentrations were 10 μM (■), 30 μM (○), 100 μM (◆); the control sample (●) contained no BCNU. Thereafter the cells were washed three times with 10 vol. of RPMI and suspended in 10 vol. of this medium at 37°. At the indicated time intervals samples were removed and assayed for glutathione.

cytes. In the range from 0 to 100 mU/ml there is a dramatic effect on the susceptibility to parasite infection whereas the effect is much less pronounced in the range between 100 mU and 1000 mU/ml (Fig. 5).

Effect of glutathione in the culture medium on parasite growth

One consequence of nitrosourea-mediated GR-inactivation is a continuous decrease of intracellular glutathione levels [6–9, 12, 21, 22] which was found to occur also in the absence of the drugs (Fig. 6). Therefore we tested if addition of glutathione to the culture medium could induce parasite growth in GR-deficient erythrocytes. Whereas glutathione disulfide (GSSG) was not effective, reduced glutathione at 5 mM had a noticeable effect (Fig. 7). At 20 mM GSH in the medium, parasite growth became independent of the GR-activity in the host cell. Although this result is consistent with the assumption that the uptake of GSH renders GR-deficient erythrocytes

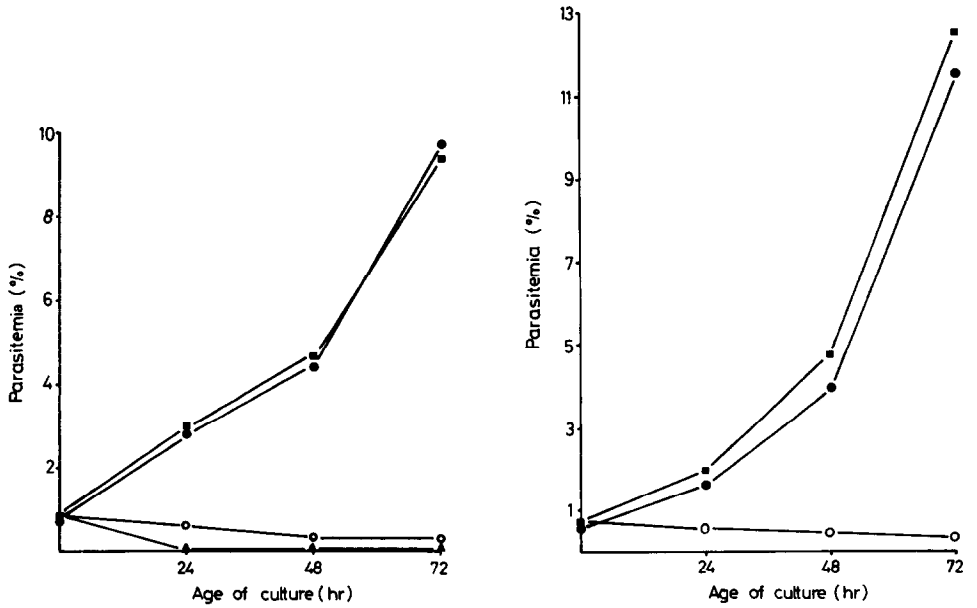

Fig. 7. Effect of glutathione on parasite growth in GR-deficient erythrocytes. The red blood cells of the control (●) contained 1000 mU GR/ml. The other erythrocytes had been pretreated with BCNU (left panel) or with HeCNU (right panel), and contained less than 20 mU GR/ml. Parasites were cultured at a hematocrit of 5% in RPMI medium with no additions (▲), in the presence of 5 mM GSH (○) and of 20 mM GSH (■). The presence of 10 mM GSSG (▲) had no effect.

normal host cells, the mode of action of extracellular GSH remains to be analyzed.

DISCUSSION

Erythrocyte glutathione reductase, in its reduced form EH_2, can be poisoned quantitatively with the nitrosourea drugs BCNU and HeCNU (Figs 1–3). Glucose, as a source of reducing equivalents, is important for complete inhibition (Fig. 1). According to studies on purified GR, a decomposition product of BCNU leads to carbamoylation of Cys58 [7, 18] whereas exposure to HeCNU alkylates this amino acid residue [11]. It should be noted that the FAD-free apoenzyme which occurs in erythrocytes [23] is possibly resistant to inactivation by nitrosoureas. This would explain the high residual activities observed after BCNU treatment when glutathione reductase was assayed in the presence of FAD [6].

As shown in Figs 4 and 5 the growth of *Plasmodium falciparum* depends on the GR activity of the host erythrocyte. It remains to be studied whether this effect on the parasite is due to the lack of GR-activity or due to the resulting loss of cellular glutathione (Fig. 6). Reduced glutathione (GSH) is believed to play an important role for the parasitation of erythrocytes [1, 26, 27]. Indeed, the addition of GSH (at unphysiologically high concentrations) to the culture medium was found to render GR-deficient erythrocytes normal host cells for parasites (Fig. 7). This aspect is further discussed in the accompanying paper [12] and elsewhere [24, 25].

In a recent paper Frischer and Ahmad [9] studied the question of how erythrocytes with various degrees of GR deficiency recover their reduced glutathione (GSH) after exposure to oxidants. They conclude that the reserve capacity of GR in red blood cells is large. Only erythrocytes that had lost more than 90% of their GR activity became functionally equivalent to glucose-6-phosphate dehydrogenase-deficient cells [9].

These findings and the results reported here suggest that the biochemistry of inherited glucose-6-phosphate dehydrogenase deficiency—including the protection from malaria [1–3, 28, 29]—can be imitated by inhibition of glutathione reductase in red blood cells.

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