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Glutathione S-transferase activity of erythrocyte genotypes HbAA, HbAS and HbSS in male volunteers administered with fansidar and quinine

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In vivo **investigations were carried out to ascertain the comparative levels of erythrocyte GST activity between non-malarious and malarious male subjects of HbAA, HbAS and HbSS erythrocyte genotypes, administered with fansidar and quinine. The incubation of human erythrocytes with 1-chloro-2,4-dinitrobenzene (CDNB) resulted in almost quantitative conjugation of glutathione (GSH) to form S-(2,4-dinitrophenyl) glutathione, determination of GST activity was carried out at 0 h and after 3, 6 and 18 h, 2 antimalarial drugs (Fansidar and quinine) were administered to the volunteers. The control values of non-malarious and malarious individuals ranged between (3.27 to 12.50 iu/gHb) and (2.75 to 12.21 iu/gHb) respectively. Erythrocyte GST activity was significantly ($p < 0.05$) lower in *P. falciparum* infected red cells compared to normal ones, except parasitized male HbSS erythrocytes. Generally, the pattern of *in vivo* erythrocyte GST activity in the presence of the 2 antimalarial drugs showed 2 phase profile. The first stage showed decreasing levels of relative GST activity within approximate time range 6 h after the drugs were administered to the volunteers. The second phase showed recovery of erythrocyte GST activity (6 - 18 h approx.) from the inhibitory effects of the drugs.**

Key words: Glutathione S-transferase (GST) activity, erythrocytes, fansidar, quinine, 1-chloro-2, 4-dinitrobenzene (CDNB).

INTRODUCTION

Fansidar^{TM} is a combination of pyrimethamine (250 mg) and sulphadoxine (50 mg) commonly used for prophylaxis and treatment of certain strains of *Plasmodium falciparum* that are resistant to chloroquine (Bray et al., 1998). This drug combination effectively blocks 2 enzymes involved in the biosynthesis of folinic acid within the parasite (Milhous et al., 1985). Quinoline blood schizontocides behave as weak bases concentrated in food vacuoles of susceptible *Plasmodia*, where they increase pH, inhibit the peroxidase activity of haem and disrupt its non-enzymatic polymerization to haemozoin. The failure to inactivate haem then kills the parasite via oxidative damage to membranes, digestive proteases and possibly other critical biomolecules of the parasite (Ducharme and Farinotti, 1996).

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A group of proteins have common ability to bind the reduced glutathione (GSH). Wide variety of hydrophobic compounds has been isolated and characterized from rat and human liver (Ketley et al., 1975, Awasthi et al., 1981; Hayes and Pulford, 1995), pigeon, locust gut, housefly and other sources (Ketley et al., 1975). These proteins have been classified based on their enzymatic activities as glutathione S-transferase (EC: 2.5.1.18) (Jacoby, 1978).

The functions of GST have been classified into 2 general categories (Harvey and Beutler, 1982) . As binding proteins (Mannervik and Danielson, 1988; Hiller et al., 2006), they function intracellularly on a broad scale in the solubilization and transport of substances such as albumin functions extracellularly (Boyer and Oslen, 1991; Oakley et al., 1999). GSTs catalyze the conjugation of electrophIlic groups of hydrophobic drugs and xenobiotics to form glutathione-thioethers (Board et al., 1990) . These thioethers are converted to mercapturic acid by the sequential actions of gamma-glutamyl transpeptidase, de-

peptidase and N-acetylase (Habig et al., 1974; Mannervik and Danielson, 1988).

GST is involved in the acquisition of drug resistance (Black and Wolf, 1991). Its role in malaria resistance has not been studied except by Dubois et al. (1995) who reported that resistance in *Plasmodium berghei* results from altered GST activity (Srivastava et al., 1999). The present study assessed the influence of antimalarial sen-sitive strain of *P. falciparum* on erythrocyte GST activity before and after the administration of fansidar and qui-nine to malarious male subjects/volunteers.

MATERIALS AND METHODS

Anti-malarial drugs

The 2 antimalarials, Fansidar^{TM} (Swiss (Swipha) Pharmaceuticals Nigeria Ltd) and quinine (BDH, UK), were purchased from Cimpok Pharmaceuticals, Amakhohia, Owerri, Nigeria.

Selection of volunteers/experimental design

15 malarious male (59 - 79 kg) subjects/volunteers each of confirmed HbAA, HbAS and HbSS genotypes between the age brackets of 21 - 34 years enrolled for this study. 9 and 6 of these volunteers received fansidar and quinine respectively. These individuals were administered with single dose of each of 2 antimalarial drugs, according to the following specifications, Fansidar [14.9 mg/kg (pyrimethamine) 2.9 mg/kg (sulphadoxine)] and quinine (5.9 mg/kg). Blood samples were then taken at time intervals of 3 h, 6 h and 18 h after dosage and analyses carried out to ascertain for erythrocyte GST activity. For comparative study, 20 apparently healthy non-malarious male (61 - 73 kg) subjects/volunteers, between 20 - 28 years were administered with the same doses of the 2 antimalarial drugs. 11 and 9 of these volunteers received fansidar and quinine respectively. Blood samples taken from these individuals in the same pattern described above for determination of this erythrocyte parameter.

Ethics

The institutional review board of the department of biochemistry, university of Port Harcourt, Port Harcourt, Rivers State, Nigeria, granted approval for this study and all subjects/volunteers involved signed an informed consent form. This conducted study was in accordance with the ethical principles that have their origins in the declaration of Helsinki. Individuals drawn were from Imo State University, Owerri, Imo State, Nigeria and environs. The research protocols were in collaboration with registered and specialized clinics and medical laboratories.

Collection of blood samples/preparation of erythrocyte haemolysate

A 5 ml of venous blood obtained from the volunteers by venipuncture was stored in EDTA anticoagulant tubes. Blood of HbSS genotype and malarious blood samples were from patients attending clinics at the Federal medical center (FMC), Imo State university teaching hospital (IMSUTH), Orlu, St.John clinic/medical diagnostic laboratories, Avigram medical diagnostic laboratories and Qualitech medical diagnostic laboratories. These centers are located in Owerri, Imo State, Nigeria.

The erythrocytes were washed by methods as described by Tsakiris et al. (2005). Within 2 h of collection of blood samples, 1.0 ml of the samples were introduced into centrifuge test tubes containing 3.0 ml of buffer solution pH 7.4: 250 mM tris (hydroxyl methyl) amino ethane–HCl (Tris-HCl)/140 mMNaCl/I.0 mMMgCl$_2$/10 mM glucose). The erythrocytes were separated from plasma by centrifugation at 1200 g for 10 min and washed 3 times by 3 similar centrifugations with the buffer solution. The erythrocytes re-suspended in 1.0 ml of this buffer were stored at 4°C. The washed erythrocytes were lysed by freezing/thawing as described by Galbraith and Watts, (1980) and Kamber et al. (1984). The erythrocyte haemolysate was used for the determination of GST activity.

Determination of erythrocytes haemolysate haemoglobin concentration

A modified method (Baure, 1980), based on cyanomethaemoglobin reaction was used for the determination of haemolysate haemoglobin concentration. The expressed values were in grams per deciliter (g/dl). A 0.05 ml portion of human red blood cell haemolysate was added to 4.95 ml of Drabkin reagent (100 mgNaCN and 300 mg $K_4Fe(CN)_6$. per liter). The mixture was left to stand for 10 min at room temperature and absorbance read at max = 540 nm against a blank. The absorbance was used to evaluate for haemolysate haemoglobin concentration by comparing the values with the standards.

Determination of erythrocyte haemolysate glutathione S-transferase activity

GST activity was assayed spectrophotometrically by monitoring the conjugation of 1- chloro-2,4dinitro benzene (CDNB) with glutathione (GSH) at max = 340 nm at 37°C (Habig et al., 1974).

The enzyme assay was according to methods of Habig et al., (1974) with minor modifications (Anosike et al. 1991). The 1.0 ml in 2% ethanol enzyme assay mixture contained 0.5 mMCDNB (0.02 ml), 1.0 mMGSH(0.05 ml), 0.68 ml of distilled water and 100 mM Phosphate buffer (K_2HPO_4/KH_2PO_4; pH = 6.5) (0.2 ml).

The CDNB was pre mixed with the phosphate buffer before use. The phosphate buffer-CDNB mixture was pre-incubated for 10 min at 37°C and the reaction started by adding GSH, followed immediately by an aliquot (0.05 ml) of the haemolysate. To the control samples, the rate of increase in absorbance at max = 340 nm was measured for 10min at 37°C against a blank solution. The enzyme assay was carried out before (control; t = 0 h) and after (tests; that is at t = 3, 6 and 18 h) the 2 antimalarial drugs were administered to the volunteers.

Statistical analyses

The experiments were designed in a completely randomized method and data collected were analyzed by the analysis of variance procedure while treatment means were separated by the least significance difference (LSD) incorporated in the statistical analysis system (SAS) package of 9.1 version.(2006).

Calculation of enzyme activity

The expression below was used to evaluate erythrocyte GST activity in international unit per gram haemoglobin (iu/gHb).

$$E_A = \frac{100}{[Hb]} \times \frac{0.D/min}{} \times \frac{V_C}{V_H}$$

Where,

E_A = Enzyme activity in iu/gHb

Table 1. *In vivo* GST activity of HbAA erythrocyte of male administered with fansidar and quinine.

DRUG	GST Activity (iu/gHb) (Relative Activity, %)		
	Time (h)	Non-malarious	Malarious
Fansidar	0	3.40+/-0.05^{a}(100.0)	2.81+/-0.76^{a}(100.0)
	3	3.02+/-0.10^{b}(88.8)	1.46+/-0.33^{b}(52.0)
	6	2.62+/-0.29^{c}(77.1)	1.17+/-0.28^{b}(41.6)
	18	3.28+/-0.24^{a,b}(96.5)	3.01+/-0.32^{a}(107.1)
Quinine	0	3.27+/-0.13^{a}(100.0)	2.52+/-0.23^{a}(100.0)
	3	1.48+/-0.16^{c}(45.3)	1.52+/-0.30^{b}(60.3)
	6	1.00+/-0.15^{d}(30.6)	1.01+/-0.16^{b}(40.1)
	18	2.89+/-0.18^{b}(88.4)	2.98+/-0.49^{a}(118.3)

Means in the column with the same letter are not significantly different at $p< 0.05$ according to LSD.

[Hb] = Haemolysate haemoglobin concentration (g/dl)
0. D/min = Change per minute in absorbance at 340 nm.
= 9.6 mM extinction coefficient of Dinitrophenyl glutathione (DNPSG).
V_C = Cuvette volume (total assay volume) = 1.0 ml.
V_H = Volume of haemolysate in the reaction system (0.05 ml).

RESULTS

Results of GST activity of 3 erythrocyte genotypes both for non-malarious and infected male subjects/volunteers at 0 h and after at 3, 6 and 18 h administration of fansidar and quinine are summarized in Tables 1, 2 and 3.

Prior to administration of the drugs the activity of GST in the 3 erythrocyte genotypes was found to increase in the following order: HbAA<HbAS<HbSS. Such tendency was showed irrespectively to malarial status of the male subjects/volunteers. However, there was no significant difference GST activity found between HbAA and HbAS erythrocyte genotypes.

Furthermore, it was found that erythrocyte GST activity significantly decreased in blood samples of *P. falciparum* infected male comparing to blood samples of non-malarious volunteers except of blood with erythrocytes of HbSS genotype. Control values of non-malarious and malarious individuals ranged between 3.27 to 12.50 iu/gHb and 2.75 to 12.2 iu/gHb, respectively. Malarious volunteers with HbAS erythrocyte genotype was found to show the lowest level of erythrocyte GST activity at 6 h after the administration of quinine (Table 2). Moreover, the highest GST activity (12.58 iu/gHb after 18 hours) was determined for malarious individuals of HbSS erythrocyte genotype (Table 3).

In summary, the pattern of *in vivo* erythrocyte GST activity when administered 2 antimalarial drugs showed 2-phase profile. The first phase showed decreasing levels of relative enzymatic activity within time range of 0 to 6 h after the drugs administering by volunteers. The second phase showed recovery of erythrocyte GST activity from the inhibitory effect of the drugs. It was shown that after 18 h from drugs consumption relative levels of GST activity of erythrocytes of infected blood were elevated even exceeding 100% (control value). Subjects of HbSS erythrocyte genotype who administered Quinine was the exception of such phenomenon.

DISCUSSION

Glutathione S-transferase (GST) activity is not the biochemical parameter routinely assayed in clinical laboratories although it could serve as essential marker for diagnostic pathology. Over- expression of GST in the erythrocytes of patients with chronic renal failure (Galli et al., 1999) and uremia (Carmagnol et al., 1981; Galli et al., 1999) is considered to be crucial parameter for the diseases prognosis.

Previous investigations by Sarin et al. (1993) showed that developing parasitaemia was accompanied by decrease of enzymatic activities in glutathione system including glutathione peroxidase (GPx), glutathione reductase (GRx) and glutathione S-transferase (GST) in the red blood cells (RBC) lysates. In the present study GST activities of erythrocyte genotypes HbAA and HbAS were found to decrease significantly when infected by *P. falciparum* comparing to corresponding RBC of blood from apparently healthy non-malarias male individuals (Tables 1 and 2). Sohail et al. (2007) showed similar results. Authors suggested decreasing GST activity to play an important role in mechanisms of host defense against malarial infection in a way of up-regulating oxidative protection. In addition, they postulated that detection of GST activity might be an essential biochemical marker for diagnostic and therapeutic goal due to malaria. Moreover, others reports have shown that certain antioxidants such as glutathione (GSH), catalase and -tocopherol were in lower levels for patients infected with malaria (Becker et al., 2004; Kavishe et al., 2006) and visceral Leishmaniasis (Neupane et al., 2008) than in control groups. Therefore, inoculation of the malarial parasites most probably

Table 2. *In vivo* GST Activity of HbAS Erythrocyte of Male Administered With Fansidar and Quinine.

DRUG	GST Activity (iu/gHb) (Relative Activity, %)		
	Time (h)	Non-malarious	Malarious
fansidar	0	4.25+/-0.10^{a}(100.0)	2.75+/-0.16^{b}(100.0)
	3	3.89+/-0.29^{a,b}(91.5)	1.96+/-0.14^{c}(71.
	6	3.84+/-0.19^{b}(90.4)	0.88+/-0.36^{d}(32.0)
	18	4.20+/-0.30^{a,b}(98.8)	3.97+/-0.65^{a}(144.4)
quinine	0	4.30+/-0.07^{a}(100.0)	2.79+/-0.11^{b}(100.0)
	3	2.66+/-0.29^{c}(61.9)	1.79+/-0.15^{c}(64.2)
	6	2.15+/-0.32^{d}(50.0)	1.76+/-0.21^{c}(63.1)
	18	3.88+/-0.25^{b}(90.2)	3.14+/-0.30^{a}(112.5)

Means in the column with the same letter are not significantly different at $p< 0.05$ according to LSD.

Table 3. *In vivo* GST activity of HbSS erythrocyte of male administered with fansidar and quinine

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DRUG	GST Activity (iu/gHb) ((Relative Activity, %)		
	Time (h)	Non-malarious	Malarious
Fansidar	0	12.50+/-1.58^{a}(100.0)	12.19+/-1.76^{a}(100.0)
	3	10.41+/-1.26^{a}(83.3)	11.37+/-0.81^{a}(93.3)
	6	10.50+/-1.56^{a}(84.0)	11.85+/-0.84^{a}(97.2)
	18	12.23+/-2.11^{a}(97.8)	12.58+/-0.17^{a}(103.2)
Quinine	0	11.65+/-1.20^{a}(100.0)	12.21+/-1.20^{a}(100.0)
	3	9.26+/-1.38^{b}(79.5)	11.63+/-0.82^{a,b}(95.2)
	6	9.89+/-1.47^{a,b}(84.9)	11.63+/-0.39^{a,b}(95.2)
	18	10.43+/-1.55^{a,b}(89.5)	11.32+/-1.13^{b}(92.7)

Means in the column with the same letter are not significantly different at $p< 0.05$ according to LSD.

initiates production of various reactive oxygen species (ROS) as a part of host defense mechanism (Becker et al., 2004) . Since there was no significant difference deter-mined in erythrocyte GST activity comparing non-mala-rias and malarias male subjects/volunteers of HbSS ge-notype (Table 3) possibly the host erythrocytes did not turn oxidative up-regulatory mechanism as a tool for con-trol and elimination of the parasite. High oxidative poten-tial of those erythrocytes as reported by Anosike et al. (1991) should be promising factor of antifecundity effect of this erythrocyte genotype.

Another reason for decreased GST activity of erythro-cytes in blood of malarias subjects based on the patho-physiology of malarial disease has been described (Du-bios et al., 1995, Liebau et al., 2002). Due to ingestion and digestion of large quantities of erythrocyte haemoglo-bin by the parasite, it subsequently has to handle high amount of potentially parasitotoxic ferroprotoporhyrin IX (FPIX) - haemin. It is known *P. falciparum* GST (pfGST) to bind to FPIX efficiently (Harwaldt et al., 2002). The un-competitive interaction was found to inhibit GST activity preferentially by binding to GST-GSH complex (Hiller et al., 2006).

In the present report comparative study of GST activity in human erythrocytes of different genotype GST showed the parameter to change in order HbAA<HbAS<HbSS (Tables 1, 2 and 3). Those data are in accordance with results of previous study of Anosike et al. (1991). Other authors reported that activation of the enzyme in HbSS and HbAS erythrocytes occur due to high levels of oxi-dants in RBC (Shalev et al., 1995). A positive activation of this redox enzyme could be determined by higher le-vels of oxidants in HbSS and HbAS than in HbAA ery-throcytes. The moderate GST activity in HbAS erythro-cytes might reflect of the hybrid nature of heterogeneous RBC (Anosike et al., 1991).

In general, the pattern of GST activity after the admini-stration of 2 antimalarial drugs was in 2 phases. First, the relative activity decreased in erythrocytes during the pe-riod of drugs uncompetitive binding to the enzyme (Man-nervik and Danielson, 1988; Ayalogu et al., 2001; Hiller et al., 2005). Second, there was the recovery phase related

to accumulation of ROS and positive activation of the enzyme should provide detoxication and neutralization of cytotoxic ROS. Consequently, the second phase is essential for homeostasis. Most probably, GST activity *in vivo* is regulated by ROS and activation of GST can be considered as an adaptive response to detoxication of cytotoxic carbonyl-, peroxide and epoxide-containing metabolites released in the cell by oxidative stress (Hayes and Pulford, 1995).

Conclusions

The present *in vivo* study showed that erythrocytes in blood of infected malarial subjects indicated significantly lower GST activity than normal RBC of HbAA and HbAS genotypes. The parameter is considered to be important for diagnosis of malaria infection. Activation of erythrocyte GST in blood of malarias male with RBC of HbAA ($p > 0.05$) and HbAS ($p < 0.05$) genotypes after the administration of 2 drugs was indicated by the parameter exceeding that in control group (100%) after 18 h. It suggested this erythrocyte property to help for monitoring of chemotherapeutic treatment in malarial disease. The findings of the present study may serve as a reliable biochemical parameter for diagnosis and a basis for monitoring therapeutic events in malarial disease.

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