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GLUTATHIONE *S*-TRANSFERASE ACTIVITY INFLUENCES BUSULFAN PHARMACOKINETICS IN PATIENTS WITH BETA THALASSEMIA MAJOR UNDERGOING BONE MARROW TRANSPLANTATION

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ABSTRACT:

Busulfan, at a dose of 16 mg/kg, is widely used in combination with cyclophosphamide as a conditioning regimen for patients undergoing bone marrow transplantation. Wide interindividual variation in busulfan kinetics and rapid clearance of the drug have been reported, especially in children. Some of the factors contributing to interpatient variability have been identified. They include circadian rhythms, age, disease, drug interaction, changes in hepatic function, and busulfan bioavailability. In this study, we demonstrate that hepatic glutathione *S*-transferase (GST) activity correlates negatively with busulfan maximum and minimum concentrations (Pearson's correlation $r = -0.74$ and -0.77, respectively) and positively with busulfan clearance (Pearson's correlation $r = 0.728$) in children with thalassemia major in the age range of 2 to 15 years. We also found that plasma alpha GST levels were 5 to 10 times higher in patients with thalassemia than in normal controls and age-matched leukemic patients, either reflecting extensive liver damage, elevated expression of the enzyme, or both in thalassemic patients. Plasma alpha GST concentrations showed a similar correlation with busulfan kinetic parameters to that observed for hepatic GST. The status of hepatic GST activity accounts, at least in part, for the observed interindividual variation in busulfan kinetics, while the observed association with plasma alpha GST is difficult to explain at present.

Busulfan is an alkylating agent that is used in combination with cyclophosphamide as a myeloablative conditioning regimen for patients undergoing bone marrow transplantation (BMT[1]) for malignant and nonmalignant disorders (Santos et al., 1983; Copelan et al., 1991; Lucarelli et al., 1995). Busulfan is metabolized extensively in the liver by glutathione *S*-transferase (GST)-mediated conjugation with glutathione (Roberts and Warwick, 1961). Cytochrome P450 enzymes seem not to be involved (Vassal et al., 1994). Alpha GST is found in the human liver at high concentrations, accounting for 5% of total soluble protein, and is mostly located in the pericentral regions (Hayes and Pulford, 1991). Recent studies (Czerwinski et al., 1996; Gibbs et al., 1996) have shown that all three known isoenzymes of GST (alpha, mu, and pi) are involved in the conjugation of busulfan in the liver. However, alpha GST is the principal catalyst of busulfan conjugation; other isoforms contribute only to a minor extent and are probably involved mainly in the protection of specific cells (Czerwinski et al., 1996). The fourth class of GST, GST theta, is known to be active toward 1,2-epoxy-3-(*p*-nitrophenoxy) propane or 1-menaphthyl sulfate. However, the role of GST theta on busulfan conjugation is not yet known. A recent report by Gibbs et al. (1999) states that young children show greater busulfan conjugating activity in intestinal biopsies, which would most likely be due to enhanced GST alpha expression. Hassan et al. (1991) suggested that the extensive interindividual variability in busulfan kinetics observed in their study might be due to the differences in levels of hepatic GSH or GST activity. We have previously reported large differences in busulfan pharmacokinetics between children with beta thalassemia major undergoing BMT (values differing by a factor of 2–12) (Poonkuzhali et al., 1999). We also noted a significant decrease in busulfan Cl/F with increasing age in this group (unpublished observations). Such a high degree of interindividual variability led us to investigate the factors that may affect busulfan metabolism. The aim of this study was to assess the effect of GST and related enzymes on busulfan pharmacokinetics in children with beta thalassemia major undergoing BMT.

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[1] Abbreviations used are: BMT, bone marrow transplantation; AUC, area under the plasma concentration-time curve; Cl/F, apparent oral clearance; GST, glutathione *S*-transferase; GSH, glutathione; ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography.

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Materials and Methods

Patients and Treatment. All children with beta thalassemia major undergoing BMT from human leukocyte antigen identical sibling donors at the Christian Medical College Hospital, Vellore, India from September 1996 to October 1998 were included in this study. Patients were randomly assigned to two groups, A and B, conditioned as follows: regimen A, 16 mg/kg busulfan

TABLE 1

Patient characteristics and busulfan dosage

UPN	Age	Sex	Weight	BSA	Bu Dose	Risk Category*
			kg	*m^2*	*mg/kg*	
29	4	F	13.5	0.65	28.4	II
95	9	F	23	0.9	23.6	III
98	2	M	15	0.6	16	II
99	5	M	15	0.65	25.6	III
100	10	M	16	0.73	16	III
103	5.5	M	18	0.73	16	III
104	4	M	14	0.60	25.1	III
105	2.5	F	10.4	0.50	27.7	II
106	10	F	28	1.00	16	III
109	13	F	33	1.09	19.9	III
110	13	F	23.8	0.88	22	III
111	5	M	14.5	0.7	28.7	III
121	2.5	M	11.2	0.51	25.7	I
122	4.5	M	14.2	0.67	28.2	I
123	2.5	M	11.1	0.55	29	II
124	4.5	M	14	0.60	16	II
125	2	F	11.3	0.52	27	I
128	12	M	25	0.96	16	III
131	5.5	F	15	0.70	16	III
133	13	M	29	1.05	16	III
134	3	M	12	0.74	16	II
136	7	F	20	0.80	16	III
139	5	F	20	0.84	16	II
140	11	M	25.3	0.95	22.7	III
142	10	M	18	0.80	16	III
143	7	M	20	0.82	24	III
144	14	M	32	1.20	16	III
151	5.5	M	15	0.62	24.5	II
157	6.5	F	19	0.84	26	III
159	15	F	26	1.12	14	III
160	13	M	30	1.10	16	III
161	3.3	M	11	0.52	29	II
162	11	F	34	1.10	16	III
163	11	M	20	0.80	16	III
165	5	F	20	0.80	16	III
166	6	F	11	0.50	16	II
167	12	M	26.9	1.00	22.6	III
168	6	F	18	0.73	24.4	II
170	5	M	17	0.66	16	III

UPN, unique patient number; Bu, busulfan.
* Risk category as per Lucarelli et al. (1990).

+ 200 mg/kg cyclophosphamide + antithymocyte globulin; regimen B, 600 mg/m^2 busulfan + 200 mg/kg cyclophosphamide.

The characteristics of the patients are given in Table 1. Of the 39 total patients, liver biopsy specimens were available for 37 patients, and samples for alpha GST assay were available for 15 patients. Forty-six normal control plasma samples (age range 18–30 years) were available for alpha GST assay. Since the patients' age range was 2 to 15 years, age-matched normal controls were not available for comparison. Instead, plasma samples from 20 age-matched patients with acute leukemias at first diagnosis (age range 2–15 years) were available. Quantitative plasma alpha GST assay was carried out using specific ELISA method as mentioned below.

Liver Biopsy Specimens. As part of the pretransplant evaluation, liver biopsy specimens were taken from all thalassemic patients for histological evaluation (approximately 2 weeks before BMT). An additional piece of tissue was obtained for the analysis of GST activity. Informed consent was obtained from the parents of all patients, and the entire study was approved by the institutional review board. Liver tissues were stored frozen (for a maximum of 1 week) at −20°C until analysis. The time interval between the collection of liver biopsy tissue and busulfan treatment ranged from 2 to 7 days.

Sonication of Liver Tissue. Liver tissue was allowed to thaw and was transferred to 1 M phosphate buffer (pH 7.4) on ice (200 μl for approximately 5 mg wet weight of tissue). It was subjected to sonication in a Soniprep sonicator (Sanyo Soniprep 150 MSE) at a temperature of 4 to 8°C. The resulting suspension was then spun at low speed (400*g*) at 4°C for 5 min to remove insoluble debris. All assays were performed immediately after sonication.

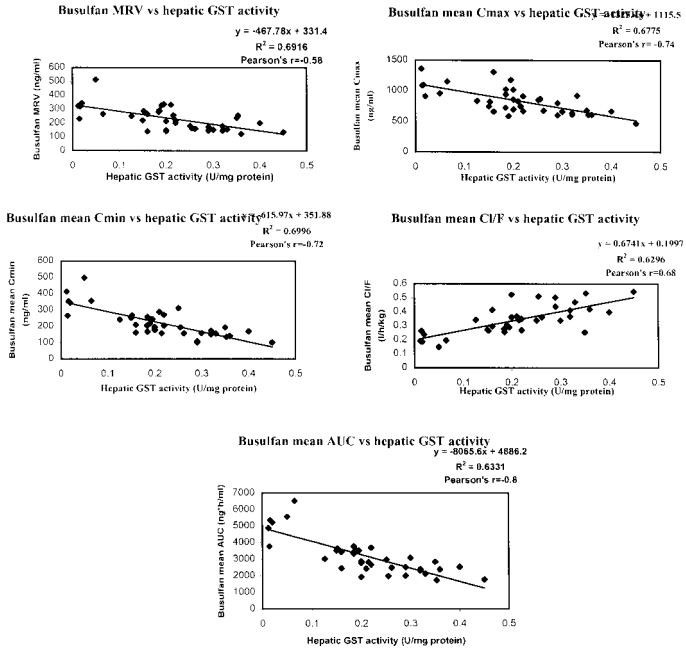

FIG. 1. *Linear regression curves for hepatic GST activity versus busulfan pharmacokinetic parameters.*

MRV, mean residual value.

GST activity [using CDNB (1-chloro-2,4-dinitro benzene) as substrate and following the increase in absorbance at 340 nm] (Awasthi et al., 1991), total glutathione levels (Owens et al., 1965), and glutathione reductase activity (Racker, 1955) in liver tissue homogenate were determined using methods described elsewhere. All enzyme activities are expressed in units per milligram of protein (specific activity). One unit of GST is the amount required to conjugate 1 μmol of the substrate with glutathione in 1 min. One unit of glutathione reductase is the amount of enzyme needed to convert 1 μmol of NADPH/min. Protein in the tissue homogenates was determined by Lowry's method (1951).

Sample Collection for ELISA. For the quantitative analysis of alpha GST, plasma samples were collected and stored at −20°C until analysis. Samples were collected from all normal controls and patients before the start of any treatment.

ELISA. The Hepkit ELISA kit (Biotrin International, Dublin, Ireland) was used (with no modification of the manufacturer's protocol) for the quantitative analysis of alpha GST. Hepkit alpha is shown to be highly specific for the detection of alpha GST. No significant cross reactivity is observed with either mu or pi isoforms of GST as determined by enzyme immunoassay or immunoblot analysis (Manning et al., 1995).

Busulfan Assay and Pharmacokinetic Analysis. Busulfan in plasma samples was determined using a new HPLC method as described before (Quernin et al., 1999). The limits of detection for the HPLC-UV method we used were 50 to 2000 ng/ml as compared with 20 to 2000 ng/ml for the gas chromatography-mass spectrometry method (Quernin et al., 1998). The sensitivity of the gas chromatography-mass spectrometry method using tetrafluorothiophenol derivatization was 10 ng/ml, and the HPLC method using the same derivatization was 20 ng/ml; the curve was linear up to a concentration of 2000 ng/ml. The interday and intraday coefficients of variation were less than 3% for the standards used to produce the calibration curve. Pharmacokinetic parameters were calculated as described elsewhere (Poonkuzhali et al., 1999), using the program Topfit (Gustav Fischer Verlag GmbH & Co., Stuttgart, Germany) (Heinzel et al., 1991).

Statistical Analysis. All statistical comparisons were performed using SPSS version 7.5 for Windows software (SPSS, Chicago, IL). Linear regression, step-wise multiple regression, Pearson's correlation analysis, and Student's *t* test were used as appropriate.

Results

The linear regression curves for hepatic GST activity versus busulfan maximum concentration (C_{max} in ng/ml), minimum concentration

TABLE 2

Correlation of hepatic and plasma glutathione S-transferase with busulfan kinetic parameters

Hepatic GST in units/mg of protein, Hepatic GSH in nmol/mg of protein, and plasma alpha GST in μg/l.

	mAUC	m Cl/F	m C_{max}	MRV	m C_{min}
Hepatic GST					
r^2 value	−0.633	0.63	−0.68	0.69	0.7
P value	0.01	0.01	0.01	0.01	0.01
Pearson's *r*	−0.80	0.728	−0.74	−0.64	−0.77
Hepatic GSH					
r^2 value	−0.248	0.28	0.09	0.17	0.12
P value	0.01	0.01	N.S.	0.05	0.05
Pearson's *r*	−0.492	0.532	−0.31	−0.417	−0.35
Plasma alpha GST					
r^2 value	−0.53	0.566	−0.42	−0.344	−0.53
Pearson's *r*	−0.70	0.751	−0.68	−0.55	−0.73
P value	0.01	0.01	0.01	0.05	0.01

m, mean; MRV, mean residual value; Pearson's *r*, correlation coefficient by Pearson's analysis; r^2 value, linear regression coefficient.

(C_{min} in ng/ml), clearance (Cl/F in l/h/kg), and area under the concentration versus time curve (AUC in ng·h/ml) are shown in Fig. 1. Significant negative correlations were found between busulfan C_{max}, C_{min}, and AUC and hepatic GST activity. There was also a similar correlation between these kinetic parameters and total GSH levels, although less significant. A significant positive correlation was found between busulfan Cl/F and total liver GST activity. Similar correlation between plasma alpha GST and busulfan kinetic parameters was also observed (Table 2). The correlation between plasma GST versus busulfan Cl/F is shown in Fig. 2. There was no significant correlation between glutathione reductase activity and any of the busulfan kinetic parameters. Overall, age and hepatic GST activity showed a significant negative correlation of −0.594 ($p < 0.01$). Hepatic GST activity and GSH levels showed a correlation of 0.324 ($p < 0.01$).

Step-wise multiple regression analysis was done with hepatic GST activity and hepatic GSH levels as possible predictors of busulfan Cl/F. In the model in which GSH entered the analysis first, there was a statistically significant improvement when hepatic GST was added to the model (r^2 change = 0.336, $p < 0.001$). When hepatic GST entered the analysis first, there was no additional improvement in the model with the addition of GSH levels (r^2 change = 0.096, $p = 0.02$).

When we included plasma alpha GST also in the step-wise multiple regression analysis, in the model where alpha GST entered the analysis first, addition of hepatic GST improved the prediction of busulfan Cl/F significantly (r^2 change = 0.331, $p = 0.001$).

We determined plasma alpha GST concentration and its relative distribution in normal controls (3.8 ± 0.525 μg/l), age-matched leukemic (4.5 ± 0.585 μg/l), and thalassemic patients (29.03 ± 11.1 μg/l) (Table 3). We compared hepatic and plasma alpha GST concentration in thalassemic patients by Pearson's correlation analysis ($r = 0.751$) (Fig. 2). Patients with high hepatic GST activity also had high plasma alpha GST concentration. Plasma alpha GST concentration in class II and class III patients were compared using two-tailed Student's *t* test. Class III patients had higher plasma alpha GST concentration than class II patients (31.5 versus 21.6 μg/l), but the difference was not statistically significant ($p = 0.14$).

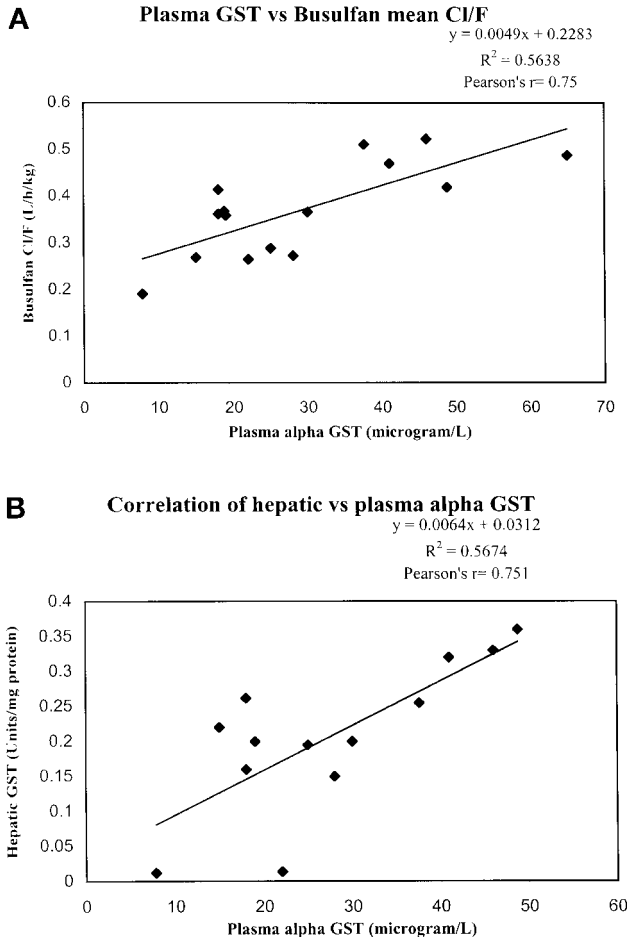

FIG. 2. *Linear regression curves for plasma alpha GST levels versus busulfan mean Cl/F (A) and hepatic GST activity (B).*

TABLE 3

Plasma alpha GST levels in thalassemic children, leukemic children, and controls

Plasma alpha GST levels of thalassemic children were significantly higher than normal controls ($p = 0.001$) and age-matched leukemic children ($p = 0.001$) when analyzed by Mann-Whitney U test.

	Thalassemic Children	Leukemic Children	Controls
Age range (yr)	2–15	2–15	18–30
n	16	20	48
Alpha GST levels (μg/l) (mean ± S.D.)	29.03 ± 11.1	4.5 ± 0.585	3.8 ± 0.525

Discussion

The metabolism of busulfan has been extensively studied in animal models (Roberts and Warwick, 1961; Vassal et al., 1994). Recent studies have shown that busulfan is metabolized in the liver by the GST (alpha, mu, and pi) enzymes (Czerwinski et al., 1996; Gibbs et al., 1996), alpha contributing to a major extent. There is no study so far reporting the role of GST theta on busulfan conjugation. Gibbs et al. (1996, 1997b) have shown in in vitro incubation with liver and intestinal cytosols that GST A1–1 is the major form involved in conjugation with busulfan in the liver and intestines. The age dependence in busulfan clearance was reported to be due to the age-dependent tetrahydrothiophene formation (Gibbs et al., 1997a) which in turn is caused by the up-regulated expression of this enzyme in young children. Recently Gibbs et al. (1999) have reported that the elevated GSH conjugation of busulfan in intestinal biopsy specimens of young children suggests that the difference in clearance is due to up-regulated busulfan-GSH conjugation in these children. These authors have stated that the possibility that GST alpha expression is up-regulated in liver of young children has not yet been assessed. The present study, although not directly showing the up-regulation of hepatic GST in these children, is the first report to show a correlation between hepatic GST activity with busulfan clearance and plasma levels. We

found a negative correlation between total GST activity and total GSH levels in the liver and the plasma concentration of busulfan in this study. This shows that busulfan pharmacokinetics in children with thalassemia are significantly influenced by hepatic GST activity. Variations in hepatic GST activity may account at least in part for the interindividual variability in the pharmacokinetics of busulfan.

There was a positive correlation of busulfan Cl/F and GST activity in this study, consistent with GST being involved in busulfan metabolism. The significant correlation between total hepatic GST activity with age (Pearson's $r = -0.594$, $p < 0.01$**) may account for the differences in busulfan clearance with age that we have observed in children with thalassemia (unpublished observations). Hassan et al. (1991) suggested that the shorter elimination half-life of busulfan in young children may be attributed to higher levels of GSH or GST such as those occurring in premature infants and neonates. Gibbs et al. (1997a) reported that tetrahydrothiophenium ion (the major metabolite of busulfan) formation in young children and adults is age-dependent. Children had 1.5 times greater area ratios for AUC [tetrahydrothiophene]/AUC [busulfan] (0–6 h) than adults, demonstrating greater capacity to metabolize busulfan by glutathione conjugation. This study confirms that hepatic GST activity, which is age-dependent, has an effect on busulfan clearance.

Recent studies (Czerwinski et al., 1996; Gibbs et al., 1996) have evaluated the role of GST isoenzymes in the conjugation of busulfan and have shown that alpha GST is the major isoform catalyzing this conjugation reaction, whereas the other forms (mu, pi, and theta) may be involved in the protection of specific cells, including hepatocytes, placenta cells, and erythrocytes. We found a significant negative correlation between plasma alpha GST levels and busulfan C_{max} and C_{min} and significant positive correlation between plasma alpha GST levels and busulfan Cl/F in this study, although the underlying reason for this association is difficult to explain at present. It has already been reported that 80% of busulfan conjugation is catalyzed by alpha GST (Gibbs et al., 1996). The observed association suggests that plasma alpha GST levels may help to predict the extent of busulfan metabolism in children with beta thalassemia major undergoing BMT. Further studies are warranted to prove this hypothesis.

In our study, children with thalassemia had approximately 10-fold higher plasma alpha GST concentrations than normal individuals (age range 18–30 years) and age-matched leukemics ($29.03 + 11.1$ versus $3.8 + 0.525$ and $4.5 + 0.585$ μg/l; $p = 0.001$), respectively. This may be explained by the fact that plasma alpha GST levels increase when there is acute (Mulder et al., 1996) or chronic liver damage (Mulder et al., 1997). Beta thalassemia major is associated with varying degrees of liver damage, which may contribute to the elevated plasma alpha GST levels in these patients. The very high plasma alpha GST levels in thalassemic patients in this study might reflect either extensive liver damage, elevated expression of the enzyme per se, or both. Previous studies have established that alpha GST is a uniquely specific and sensitive marker of damage to hepatocytes (Trull et al., 1994; Nelson et al., 1995; Vaubourdolle et al., 1995). Its rapid release into and removal from the circulation provides more immediate information about liver status than other conventional aminotransferase markers (Mulder et al., 1997). Recently, alpha GST levels in the plasma and liver tissues of healthy organ donors have been reported (Mulder et al., 1999). This study is the first to report alpha GST levels in children with beta thalassemia major.

The correlation between total hepatic GST activity with plasma alpha GST concentration in thalassemic patients in this study suggests a possibility of plasma alpha GST as one of the markers or probable determinants of busulfan metabolism in this group of patients; however, further studies are needed to confirm this hypothesis.

The higher mean plasma alpha GST levels in class III than class II patients (31.5 μg/l in class III versus 21.6 μg/l in class II), although not statistically significant, confirm that this can be a good marker of liver status in patients with thalassemia major.

The correlation of hepatic GST activity and plasma alpha GST levels with busulfan kinetic parameters reported in this study offers one explanation for the large interindividual differences in busulfan metabolism observed in children with thalassemia major.

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