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The effect of treatment with high dose melphalan, cisplatin or carboplatin on levels of glutathione in plasma, erythrocytes, mononuclear cells and urine

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Abstract Glutathione (GSH) has been implicated as an important factor in the detoxification of many electrophilic xenobiotics, including agents used in cytotoxic chemotherapy. Maintenance of high levels of GSH in normal tissues is believed to be important in the prevention of drug-induced toxicity. Previous studies have indicated that exposure of cells to some toxic electrophiles both *in vitro* and *in vivo* can cause a temporary decrease in intracellular levels of GSH. In this paper we report that in a series of 22 children and young adults treated with high dose melphalan (ten courses studied, all 200 mg/m^2), cisplatin (eight courses, 80–104 mg/m^2) or carboplatin (seven courses, 507–750 mg/m^2) there was no significant alteration in the level of plasma, erythrocyte or urine GSH in the period immediately following drug administration. Fluctuations in the level of GSH in mononuclear cells were observed in some patients but did not follow any consistent pattern and were similar to those observed in a series of nine normal adult controls over the same time course. These results suggest that for melphalan, cisplatin and carboplatin, drug-GSH adduct formation is insufficient to cause a measurable decrease in intracellular GSH levels in normal peripheral haematopoietic cells during the course of treatment.

Key words Glutathione · Cytotoxic chemotherapy · Cisplatin · Carboplatin · Melphalan · Nephrotoxicity

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Introduction

Glutathione (GSH) is the most abundant non-protein intracellular thiol and, as such, is an essential component of the system which maintains a reducing environment within the cell. In addition, GSH is involved in the detoxification of a wide range of electrophilic xenobiotics through the formation of inactive conjugates, which may be subsequently metabolised and excreted as mercapturic acids [1]. An elevation in the level of cytoplasmic GSH has been implicated in the development of tumour resistance to a number of drugs used in the treatment of cancer, including the bifunctional alkylating agent, melphalan [2], and the platinum compound, cisplatin [3], and also appears to play an important role in the protection of normal tissues against drug-induced damage, including cisplatin-induced nephrotoxicity. Administration of glutathione or glutathione esters has been shown to reduce cisplatin-induced kidney damage in both animal [4] and clinical [5, 6] studies. Conversely, depletion of GSH by the administration of buthionine sulfoximine aggravates cisplatin-induced nephrotoxicity in mice [7] and young rats [8].

For several compounds, such as menadione [9] and diethyl maleate [10], the formation of GSH adducts can lead to a significant decrease in intracellular GSH, which, in turn, may allow cellular damage through the unopposed action of oxygen free radicals. It has been shown that exposure of kidney cortical slices [11] or a mouse mammary tumour cell line [12] to cisplatin *in vitro* results in glutathione depletion but the effects of this drug on GSH levels *in vivo* have not been studied.

Treatment of mice with cyclophosphamide has been shown to cause an initial reduction in GSH levels in the bone marrow, followed 5–7 days later by a rebound increase [13]. It has been suggested that this phenomenon may underlie the observation that "priming" mice with a low dose of cyclophosphamide will protect them

against a subsequent high dose [14], an approach which has been exploited clinically in patients receiving high dose melphalan [15].

Lind et al. [16] demonstrated that treatment of lymphoid cells *in vitro* with 4-hydroperoxifosfamide or chloroacetaldehyde caused a marked reduction in GSH levels, presumably due to the formation of adducts with the drug or its metabolites. A similar depletion was observed in the lymphocytes of a patient undergoing treatment with ifosfamide, but the effect of other electrophilic drugs on GSH levels in patients has not been extensively reported.

We have previously reported that patients treated with a combination of high dose melphalan and carboplatin prior to bone marrow transplantation experienced severe nephrotoxicity [17]. This was unexpected in view of the fact that these drugs are associated with minimal nephrotoxicity when administered alone, and may be related to the dose intensity of the schedule employed, as renal toxicity was not observed when the same drugs were given over a longer period [18]. In view of the evidence that nephrotoxicity associated with cisplatin has been linked with GSH depletion in renal tubular cells, we postulated that the combination of melphalan and carboplatin may produce toxicity by an additive effect on intracellular GSH levels. In order to test this hypothesis, and to establish if drugs apart from the oxazophophorenes may affect GSH homeostasis, we studied the effect of chemotherapy on GSH levels in the peripheral mononuclear cells, erythrocytes, plasma and urine of patients undergoing treatment with cisplatin, carboplatin or melphalan.

Materials and methods

Patients and samples

Twenty-two children and young adults (age 2–20 years) with a variety of malignancies were used as subjects for this study. Clinical details are given in Table 1. Two patients with neuroblastoma (Table 1, numbers 13 and 15) were studied on three separate occasions, when undergoing treatment with cisplatin, carboplatin or melphalan. Blood samples were collected from a central venous catheter at timed intervals following the start of the administration of melphalan (200 mg/m^2, nine patients studied) given as an intravenous bolus, cisplatin (80–104 mg/m^2, eight patients studied), given as an intravenous infusion over 24 h or carboplatin (507–750 mg/m^2, seven patients studied), given as an intravenous infusion over 1 h. The protocol was approved by the local ethics committee.

Serial samples were also taken from a peripheral venous catheter in nine normal adult volunteers in order to document normal diurnal fluctuations in GSH levels in plasma, erythrocytes and mononuclear cells.

Chemicals

Monobromobimane was obtained from Novabiochem (Nottingham, UK). All other chemicals were obtained from either Sigma (Poole, UK) or FSA (Loughborough, UK) and were of the highest purity available.

Table 1 Clinical details of patients studied

Patient number	Age (years)	Diagnosis	Drugs	Dose (mg/m^2)
1	20	Ewing's sarcoma	Melphalan	200
2	2	Glioma	Carboplatin	585
3	8	Glioma	Carboplatin	532
4	10	Hodgkin's disease	Melphalan	200
5	13	Intracranial teratoma	Carboplatin	558
6	18	Malignant fibrous astrocytoma	Cisplatin	104
7	5	Medulloblastoma	Carboplatin	507
8	6	Mucoepidermoid carcinoma	Cisplatin	100
9	4	Neuroblastoma	Carboplatin	750
10	4	Neuroblastoma	Melphalan	200
11	5	Neuroblastoma	Carboplatin	709
12	5	Neuroblastoma	Melphalan	200
13	5	Neuroblastoma	Carboplatin	750
			Cisplatin	80
			Melphalan	200
14	6	Neuroblastoma	Melphalan	200
15	8	Neuroblastoma	Carboplatin	718
			Cisplatin	80
			Melphalan	200
16	5	Osteogenic sarcoma	Cisplatin	103
17	10	Osteogenic sarcoma	Cisplatin	104
18	10	Osteogenic sarcoma	Cisplatin	103
19	20	Osteogenic sarcoma	Cisplatin	103
20	5	Rhabdomyosarcoma	Melphalan	200
21	9	Rhabdomyosarcoma	Melphalan	200
22	3	Triton tumour	Melphalan	200

Determination of glutathione levels

GSH levels in erythrocytes, mononuclear cells and plasma were measured by the method of Colgreave and Moldeus [19] with minor alterations. Samples (5 ml) were collected into pre-cooled tubes containing lithium heparin and processed immediately to minimise errors caused by GSH oxidation or breakdown [20]. For the analysis of plasma GSH, 0.5 ml of whole blood was centrifuged at 13 000 rpm for 1 min in a microcentrifuge. One hundred and fifty microlitres of plasma was added to an equal volume of 8 mM monobromobimane in 50 mM N-ethylmaleimide, pH 8.0. Fifteen microlitres of 100% (w/v) trichloroacetic acid (TCA) was added after 5 min incubation in the dark and the sample stored at − 80°C prior to analysis.

For the measurement of erythrocyte GSH, 0.4 ml of whole blood was mixed with an equal volume of phosphate-buffered saline (PBS) and passed down a 3-ml column of a slurry consisting of 50% (w/w) microcrystalline cellulose and 50% α-cellulose in order to remove white blood cells. Cells were washed in 20 ml of PBs and resuspended in 10 ml of PBS. An aliquot was mixed with monobromobimane and TCA as described above. Cells were counted using a Coulter counter.

For the measurement of GSH in mononuclear cells, whole blood was separated over Lymphoprep (Nycomed, Oslo, Norway) and washed in PBS. Red cells were lysed by incubation in a solution containing 0.83% (w/v) NH_4Cl, 0.037% (w/v) EDTA and 0.1% $KHCO_3$ for 4 min prior to resuspension in 0.4 ml of PBS. Cells were counted using a haemocytometer and viability assessed by Trypan Blue exclusion. The suspension was mixed with monobromobimane and TCA as described above.

For the estimation of the amount of derivitised GSH, samples were centrifuged at 13 000 rpm for 10 min using a microcentrifuge and 25 µl aliquots of the supernatant applied to a 5 µm Spherisorb ODS column (4.6 × 150 mm, Jones Chromatography, Hengoed, UK) equilibrated with 10% acetonitrile, 0.25% acetic acid (solvent A). High pressure liquid chromatography (HPLC) was performed using a Waters model 625 pump and system controller (Millipore, Watford, UK). Peaks were detected using a Waters model 470 fluorescence detector set at an excitation wavelength of 394 nm and measuring at 480 nm. The flow rate was 1 ml/min. After 5-min isocratic flow, elution of derivitised GSH was achieved using 75% acetonitrile, 0.25% acetic acid (solvent B) using a convex gradient (curve 6) over 8 min followed by a 4-min flow with solvent B. GSH concentrations were estimated from a calibration curve generated using reduced glutathione mixed with 1.5 mM dithiothreitol. The lower limit of detection using this assay was found to be 0.5 µmol/l.

Monobromobimane derivitisation could not be used for the determination of levels of GSH in urine due to the presence of interfering peaks during HPLC. For these samples orthophthaldehyde (OPA) was used as described by Neuschwander-Tetri and Roll [21], to measure total (i.e. oxidised and reduced) GSH. A 100-µl portion of urine was mixed with an equal volume of 25 mM dithiothreitol and 50 µl of 0.1 M TRIS-HCl pH 8.5. After incubation for 30 min on ice, proteins were precipitated by the addition of 750 µl of 2.5% (w/v) sulphosalicylic acid and samples centrifuged for 5 min at 13 000 rpm in a microcentrifuge. A 200-µl aliquot of the supernatant was mixed with an equal volume of 32 mM OPA in 0.4 M potassium borate pH 9.9. After incubation at room temperature for 1 min 2 ml of 100 mM sodium phosphate pH 7 was added and the samples centrifuged for 10 min at 13 000 rpm in a microcentrifuge. A 100-µl sample of the supernatant was applied to a 5-µm Spherisorb ODS column (4.6 × 150 mm, Jones Chromatography, Hengoed, UK) equilibrated in 0 15 M sodium acetate pH 7 with 6% (v/v) methanol Derivitised products were eluted with a 6–100% linear gradient of methanol using the HPLC apparatus described above Peaks were detected using an excitation wavelength of 338 nm and an emission wavelength of 425 nm. The lower limit of detection for this assay was found to be 10 µmol/l.

Results

Mean plasma, erythrocyte and mononuclear cell GSH levels in the initial samples from the normal adults and of the patients undergoing chemotherapy are given in Table 2. The fluctuation in these levels following the initial samples are shown in Fig. 1, and the maximum and minimum values obtained shown in Table 3.

The GSH level in plasma (1.19 µmol/l) and erythrocytes (0.26 fmol/cell) in the normal adults was similar to that in the patients prior to treatment with cytotoxic drugs. As shown in Table, the level in peripheral mononuclear cells (1.26 fmol/cell) was similar to that in patients prior to treatment with cisplatin (1.46 fmol/cell) or carboplatin (1.57 fmol/cell) but the average value for the patients due to receive melphalan tended to be higher (2.05 fmol/cell), however this difference did not achieve statistical significance.

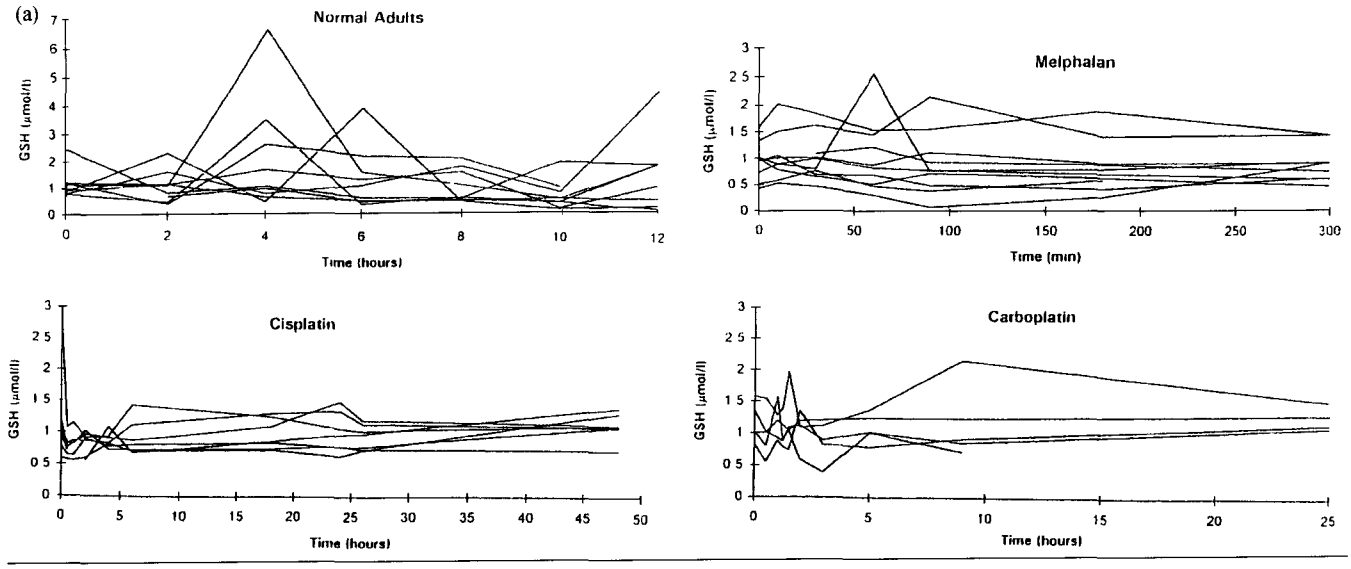
In three of the normal controls, the GSH level in one plasma sample was more than 3 times the baseline value, but this rise did not occur at a consistent time during the course of the study. The level of fluctuation was far less marked in the patients undergoing chemotherapy. No consistent pattern of fluctuation was seen in the GSH levels of erythrocytes or peripheral mononuclear cells in either the normal adults studied or the

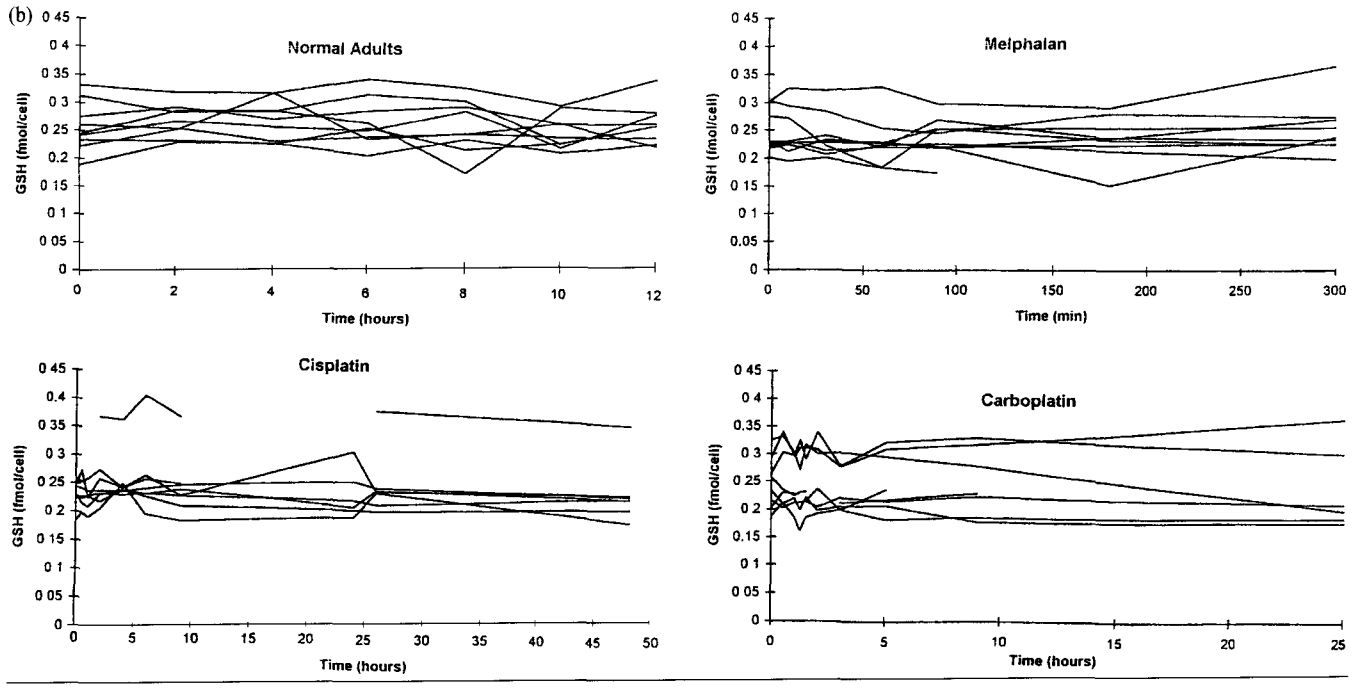
Table 3 Levels of glutathione in normal controls and patients; Maximum and minimum values measured during period of study

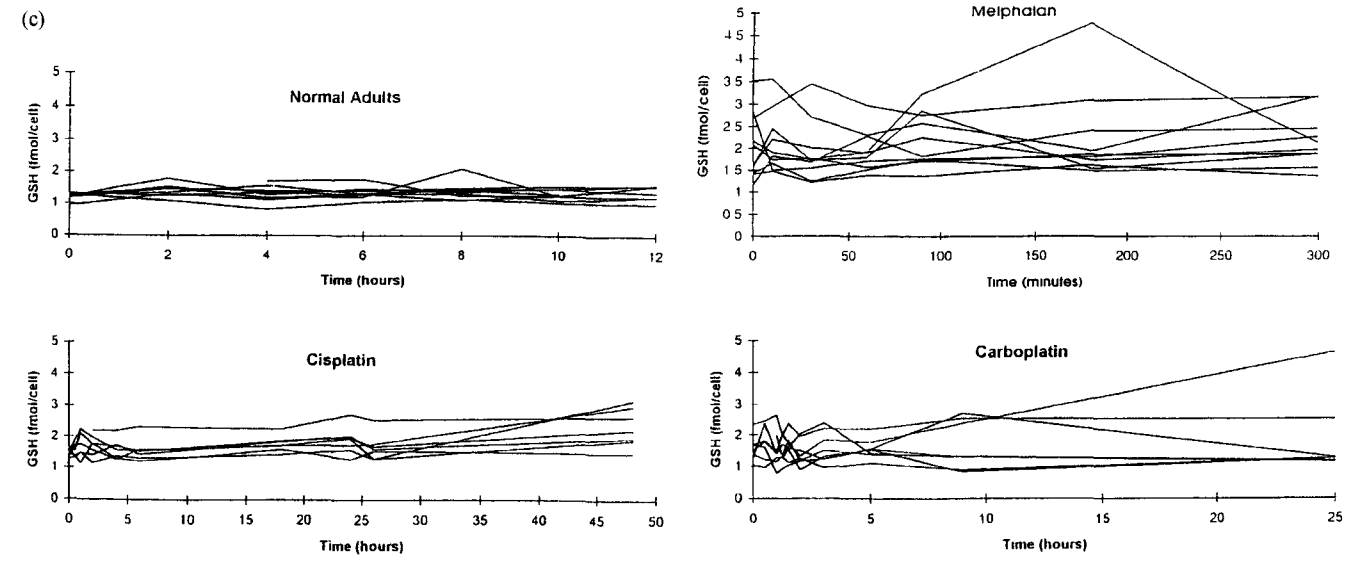
Subjects	Plasma (µmol/l)	Erythrocytes (fmol/cell)	Mononuclear cells (fmol/cell)
Normal adults	0.35–6 77	0 18–0.35	0.83–2 11
Melphalan-treated	0.08–2.57	0.15–0.37	1.21–4 76
Cisplatin-treated	0.56–1.49	0.19–0.41	1.01–3.14
Carboplatin-treated	0.40–2.16	0.16–0.37	0 79–4.60

Table 2 Initial levels of glutathione in normal controls and patients. Values are given as the mean ± SD. Figures in brackets refer to the number of subjects studied

Subjects	Plasma (µmol/l)	Erythrocytes (fmol/cell)	Mononuclear cells (fmol/cell)
Normal adults	1.19 ± 0.55 (8)	0.26 ± 0.04 (9)	1.26 ± 0.17 (9)
Melphalan-treated	0.94 ± 0.35 (10)	0.24 ± 0.04 (10)	2.05 ± 0 76 (10)
Cisplatin-treated	1.20 ± 0.72 (7)	0 23 ± 0.02 (7)	1.46 ± 0.14 (7)
Carboplatin-treated	1.15 ± 0.30 (5)	0.25 ± 0.5 (8)	1.57 ± 0.39 (8)


(a)
Normal Adults
GSH (µmol/l)
Time (hours)
Melphalan
GSH (µmol/l)
Time (min)
Cisplatin
GSH (µmol/l)
Time (hours)
Carboplatin
GSH (µmol/l)
Time (hours)


(b)
Normal Adults
GSH (fmol/cell)
Time (hours)
Melphalan
GSH (fmol/cell)
Time (min)
Cisplatin
GSH (fmol/cell)
Time (hours)
Carboplatin
GSH (fmol/cell)
Time (hours)


(c)
Normal Adults
GSH (fmol/cell)
Time (hours)
Melphalan
GSH (fmol/cell)
Time (minutes)
Cisplatin
GSH (fmol/cell)
Time (hours)
Carboplatin
GSH (fmol/cell)
Time (hours)

patients undergoing chemotherapy. In particular, there was no evidence for an alteration in GSH levels immediately following the onset of chemotherapy or after its completion (see Table 3 and Fig. 1).

Urine was obtained from 19 patients during the course of treatment (7 treated with melphalan, 4 with carboplatin and 8 with cisplatin). No glutathione (oxidised or reduced) was detected in any of the samples analysed.

Discussion

The role of GSH in the protection of cells against damage caused by oxygen free radicals and toxic xenobiotics is well established (for review see [22]), and an elevation in cytoplasmic levels is frequently found in cells grown in culture which are resistant to bifunctional alkylating agents [2, 23] or platinum-containing drugs [24]. Elevations have been associated with an increase in either the level of the rate limiting enzyme in the GSH synthesis pathway, γ-glutamyl cysteine synthetase [25], or an increase in surface membrane-bound γ-glutamyl transpeptidase [26], an enzyme which is capable of salvaging GSH from the surrounding milieu.

As well as a stable elevation associated with drug resistance, Barranco et al. [27] demonstrated that acute changes may also occur following exposure to melphalan *in vitro*. In this experiment, exposure of a human gastric carcinoma cell line to a 99% lethal dose of melphalan caused a 25% rise in GSH in melphalan-sensitive and a 150% rise in GSH in melphalan-resistant cells following drug exposure. In the drug-resistant cells, a 50% rise was seen within 6 h. No evidence was seen in these studies of a fall in GSH levels following drug exposure. Conversely, Lind et al. [16] noted that exposure of a lymphoid cell line to ifosfamide *in vitro*, or peripheral mononuclear cells *in vivo*, caused a transitory decrease in intracellular GSH.

As well as being implicated in the development of drug resistance in malignant cells, glutathione appears to play an important part in the reduction of toxicity in normal tissues. For example, several studies have been reported which implicate glutathione depletion in the pathogenesis of cisplatin-induced nephrotoxicity. This effect may be exacerbated by prior depletion of GSH by buthionine sulfoximine, an inhibitor of γ-glutamyl cysteine synthetase [7], and ameliorated by treatment with intravenous GSH [28]. Melphalan and carboplatin are normally associated with minimal nephrotoxicity but, in a recent trial in children with cancer, the combination of these two agents in high doses produced severe nephrotoxicity [17]. We postulated that treatment with these agents may potentiate nephrotoxicity through the depletion of intracellular GSH and, therefore, measured levels in patients in the period immediately prior to and following drug exposure.

Samples of blood were processed immediately following withdrawal from a central venous line in the case of the patients and a peripheral catheter in the normal adults. Using monobromobimane it is possible to derivatise GSH in intact cells, thereby minimising possible oxidation after cell lysis. Unlike monochlorobimane, conjugation of monobromobimane to GSH occurs spontaneously in the absence of glutathione S-transferase. Marked fluctuations were noted in some of the plasma values obtained from the normal adults, probably reflecting a small degree of haemolysis associated with the difficulty of maintaining a patent peripheral catheter over a prolonged period. Disregarding these probably artifactual results, the values obtained in all the groups studied was similar to those previously reported for plasma 29–31], red blood cells [32, 33] and mononuclear cells [34]. The levels in the white blood cells of patients undergoing treatment with high dose melphalan were higher than those from the other groups of patients. Although this difference did not achieve statistical significance, the number of patients studied was small. The elevation may reflect the fact that this group received 300 mg/m^2 of cyclophosphamide as "priming" 7 days before treatment with melphalan, a procedure which has been associated with a delayed rise in GSH levels in bone marrow in mice [13].

Although treatment with melphalan, cisplatin and carboplatin does not appear to cause a significant effect on GSH levels in peripheral mononuclear cells, alterations cannot be excluded in the principle targets of their toxic effects, the bone marrow in the case of melphalan and carboplatin and renal tubular cells in the case of cisplatin. However, these results do suggest that GSH depletion is not a universal cellular response to treatment with electrophilic drugs *in vivo*. In studies of drug-DNA adduct formation it has been demonstrated that both melphalan [35] and cisplatin [36] are capable of crossing the plasma membrane of peripheral mononuclear cells. This suggests that the lack of change of GSH levels in mononuclear cells is not due to an inability of the drugs to penetrate the cell membrane. Direct studies of the effect of treatment on GSH homeostasis in the kidney are precluded by the difficulty of obtaining fresh biopsy material.

Lind et al. [16] noted a depletion in GSH levels in peripheral mononuclear cells following treatment with

Fig. 1a Changes in plasma glutathione (*GSH*) levels with time for normal adults and patients treated with melphalan, cisplatin or carboplatin. **b** Changes in red blood cell GSH levels with time for normal adults and patients treated with melphalan, cisplatin or carboplatin. **c** Changes in peripheral mononuclear cell GSH levels with time for normal adults and patients treated with melphalan, cisplatin or carboplatin

ifosfamide, a drug associated with considerable nephrotoxicity. The dose of drug used (5 g/m^2) and, thus, the amount of metabolites produced in this patient, was much greater than that used in patients treated with cisplatin, melphalan or carboplatin. On stoichiometric grounds, depletion of GSH secondary to adduct formation is less likely to be measurable in the subjects of our study than in those treated with oxazophosphorenes.

Treatment of experimental animals with potent inhibitors of γ-glutamyl transpeptidase activity has been shown to cause a marked increase in levels of GSH in the urine due to a decrease in tubular reabsorption [37]. Although cisplatin has been shown to act as an inhibitor of γ-glutamyl transpeptidase, both *in vitro* and *in vivo* [38], we could demonstrate no increase in GSH loss in patients undergoing treatment with the drug.

In summary, in this study we have demonstrated that peripheral mononuclear and red blood cells are able to maintain normal levels of cytoplasmic GSH levels following exposure to cisplatin, carboplatin or high dose melphalan in children and young adults undergoing treatment for cancer. In addition, such treatment has no detectable effect on the ability of the kidney to reabsorb filtered GSH. These results suggest that these drugs do not have a generalised effect on GSH homeostasis, although effects on specific organs such as the kidney cannot be excluded.

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